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The role of the septo-hippocampal system in fear and anxiety.

By

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Aldemar Bas Degroot

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirement for the degree of Doctor of Philosophy.

Department of Psychology

Edmonton, Alberta

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University of Alberta

Faculty of Graduate Studies and Research

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Abstract

In this dissertation, the role of the septo-hippocampal system in anxiety modulation was explored. Two animal models of rat anxiety were used: the elevated plus-maze and shock-probe burying tests. A reduction in anxiety in the elevated plus-maze is indicated by increased open-arm exploration, whereas a reduction in anxiety in the shock-probe burying test is indicated by decreased burying behavior or increased contacts with the shock-probe (chapter 1). In the first series of experiments (chapter 2), reversible tetrodotoxin (TTX) inactivation of the dorsal hippocampus, ventral hippocampus, septal region, or fimbria fornix induced anxiolytic-like effects that vary in nature. More specifically, fimbria fornix inactivation increased both open arm exploration and the number of contact-induced shocks from the probe, while having no effect on burying behavior. Both septal and ventral hippocampal inactivation increased open arm exploration and decreased burying behavior, but had no effect on the number of probe-shocks. In contrast, TTX inactivation of the dorsal hippocampus increased the number of shocks taken by the rats, but did not affect open arm activity or burying behavior. The data suggest that the control of specific anxiety reactions is functionally segregated within different aspects of the septo-hippocampal system. In the second series of experiments (chapter 3), microinfusions (10 μg/0.5 μl) of the acetylcholinesterase inhibitor physostigmine in either the dorsal or the ventral hippocampus increased rats' open-arm exploration in the plus-maze test, and decreased burying behavior in the shock-probe test. Interestingly, infusions in the ventral, but not the dorsal hippocampus also increased the number of contacts rats made with the shock-probe. In the third series of experiments (chapter 4), microinfusions (20 ng/0.4 ul) of the $GABA_A$ agonist muscimol

into either the lateral or the medial septum increased rats' open-arm exploration in the plus-maze test, and decreased their burying behavior in the shock-probe test. More importantly, the final set of experiments (chapter 5) showed that the combination of sub-effective doses of physostigmine in the hippocampus (5 ug) and muscimol in the medial septum (2.5 ng) significantly reduced burying of the shock probe, suggesting that the septal GABAergic and hippocampal cholinergic systems may act synergistically in the modulation of anxiety.

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First and foremost, I would like to express gratitude to my supervisor, Dr. Dallas Treit for his academic instruction, his inspiration, and for giving me the opportunity to work in his laboratory. Dr. Treit was always available to give me his time and advice and made my Ph.D. an enjoyable experience. In addition I would like to thank my committee members Dr. Clayton Dickson and Dr. Frederick Colbourne for providing advice, insight and time. Furthermore I thank Dr. Peter Hurd and Dr. Glen Baker for their time and effort. Lastly, I would like to thank Dr Robert Rodgers for his willingness to be my external committee member.

1

Table of Contents

Chapter 1: Introduction 1

Background 2

Animal models of anxiety 2

The elevated plus maze 3

The shock-probe burying test 4

The septum, hippocampus, and anxiety 6

Septum and hippocampus: anatomy and neurochemistry 7

The GABAergic and cholinergic systems and anxiety 11

Thesis rationale 14

References 22

Chapter 2: Anxiety is functionally segregated within the septo-hippocampal system 40

Introduction 41

Materials and methods 42

Results (Experiment 1) 50

Results (Experiment 2) 51

Results (Experiment 3) 52

Results (Experiment 4) 53

Results (Experiment 5) 54

Discussion 54

References 85

Chapter 3: Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests 93

Introduction 94

Materials and methods (Experiment 1) 97

Materials and methods (Experiment 2) 102

Results (Experiment 1) 103

Results (Experiment 2) 104

Discussion 106

References 126

Chapter 4: Septal GABAergic and Hippocampal Cholinergic Systems Modulate Anxiety in the Plus-maze and Shock-probe Tests 137

Introduction 138

Materials and methods (Experiment 1) 141

Results (Experiment 1) 146

Materials and methods (Experiment 2) 148

Results (Experiment 2) 149

Discussion 151

References 171

Chapter 5: Septal GABAergic and hippocampal cholinergic systems interact in the modulation of anxiety. 180

Introduction 181

Materials and methods (Experiment 1) 184

Materials and methods (Experiment 2) 188

Materials and methods (Experiment 3) 189

Results (Experiment 1) 190

Results (Experiment 2) 191

Results (Experiment 3) 191

Discussion 192

References 212

Chapter 6: Discussion 222

General Discussion 223

Limitations 226

Future directions and conclusions 228

References 233

List of Tables

Table 2-1: Mean (+/- S.E.M.) Percentage Open Arm Exploration, Total Arm Entries and Closed Arm Entries in the Plus-maze Task, and Mean (+/- S.E.M.) Activity and Reactivity in the Shock-probe Burying Test........................ 58

Table 3-1: Mean (+/- S.E.M.) Total Arm Entries and Closed Arm Entries in the Plus-maze Task, and Mean (+/- S.E.M.) Activity and Reactivity in the Shock-probe Burying Test 111

Table 4-1: Mean (+/- S.E.M.) Total Arm Entries in the Plus-maze Task, and Mean (+/- S.E.M.) Activity and Reactivity in the Shock-probe Burying Test 155

Table 4-2: Mean (+/- S.E.M.) Total Arm Entries in the Plus-maze Task, and Mean (+/- S.E.M.) Activity and Reactivity in the Shock-probe Burying Test 156

Table 5-1: Mean (+/- S.E.M.) Activity, Reactivity, and Burying Scores in the Shock-probe Burying Test.. 197

Table 5-2: Mean (+/- S.E.M.) Activity, Reactivity, and Burying Scores in the Shock-probe Burying Test.. 198

Table 5-3: Mean (+/- S.E.M.) Activity, Reactivity, and Burying Scores in the Shock-probe Burying Test.. 199

Table 6-1: Summary Table 232

List of Figures

Figure 1-1: The Elevated Plus-Maze. 16

Figure 1-2: The Shock-Probe Burying Test.. 17

Figure 1-3: Schematic representation of the neuroanatomy and neurochemistry of the septo-hippocampal system.. 19

Figure 1-4: Schematic representation of afferent and efferent connections of the septum and the hippocampus.. 21

Figure 2-1: Schematic illustration of coronal sections of the rat brain showing the approximate location of fimbria fornix infusion sites. 60

Figure 2-2: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of TTX (5 ng/0.5 µl) or vehicle (0.5 µl) into the fimbria fornix. 62

Figure 2-3: Number of shocks in the shock probe apparatus after infusions of TTX (5 ng/0.5 µl) into the fimbria fornix.. 64

Figure 2-4: Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septum infusion sites. 66

Figure 2-5: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of TTX (5 ng/0.5 μl) or vehicle (0.5 μl) into the medial septum. 68

Figure 2-6: Bury time (LOG; s) in the shock probe apparatus after infusions of TTX (5 ng/0.5 μl) into the medial septum...................................... 70

Figure 2-7: Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites. 72

Figure 2-8: Number of shocks in the shock probe apparatus after infusions of TTX (5 ng/0.5 μl) into the dorsal hippocampus.................................... 74

Figure 2-9: Schematic illustration of coronal sections of the rat brain showing the approximate location of ventral hippocampal infusion sites. 76

Figure 2-10: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of TTX (5 ng/0.5 μl) or vehicle (0.5 μl) into the ventral hippocampus 78

Figure 2-11: Bury time (LOG; s) in the shock probe apparatus after infusions of TTX (5 ng/0.5 μl) into the ventral hippocampus 80

Figure 2-12: Labeled cell bodies and fibers following BDA injections in the dorsal hippocampus (A). There were no terminal regions in the amygdala following Injections of BDA in the dorsal hippocampus (B)...................... 82

Figure 2-13: Labeled cell bodies and fibers following BDA injections in the ventral hippocampus (A). Densely labeled fibers in the amygdala were observed following Injections of BDA in the ventral hippocampus (B)..................... 84

Figure 3-1: Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites. 113

Figure 3-2: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of physostigmine (10 ug/0.5 μl) or vehicle (0.5 μl) into the dorsal hippocampus. 115

Figure 3-3: Bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (10 ug/0.5 μl) into the dorsal hippocampus 117

Figure 3-4: Schematic illustration of coronal sections of the rat brain showing the approximate location of ventral hippocampal infusion sites. 119

Figure 3-5: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of physostigmine (10 ug/0.5 μl) or vehicle (0.5 μl) into the ventral hippocampus 121

Figure 3-6: Bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (10 ug/0.5 μl) into the ventral hippocampus.................. 123

Figure 3-7: Number of shocks in the shock probe apparatus after infusions of physostigmine (10 ug/0.5 μl) into the ventral hippocampus........................ 125

Figure 4-1: Schematic illustration of coronal sections of the rat brain showing the approximate location of A) medial septal and B) lateral septal infusion sites. 158

Figure 4-2: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of muscimol (20 ng/0.4 μl) or vehicle (0.4 μl) into the medial or lateral septum. 160

Figure 4-3: Bury time (SQR; s) in the shock probe apparatus after infusions of muscimol (20 ng/0.4 μl) or vehicle (0.4 μl) into the medial or lateral septum. 162

Figure 4-4: Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septal infusion sites. 164

Figure 4-5: Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites. 166

Figure 4-6: Percentage of open arm entries (A) and percentage of open arm time (B) after infusions of muscimol (20 ng/0.4 µl) into the medial septum, physostigmine (10 µg/1 µl) into the dorsal hippocampus, or combined infusions... 168

Figure 4-7: Bury time (SQR; s) in the shock probe apparatus after infusions of muscimol (20 ng/0.4 µl) into the medial septum, physostigmine (10 µg/1 µl) into the dorsal hippocampus, or combined infusions................... 170

Figure 5-1: Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septum infusion sites. 201

Figure 5-2: Bury time (LOG; s) in the shock probe apparatus after infusions of muscimol (2.5, 5, or 10 ng/0.4 µl) or vehicle (0.4 µl) into the medial septum.. 203

Figure 5-3: Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites. 205

Figure 5-4: Bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (5, 10, or 20 ug/1 µl) or vehicle (1 µl) into the dorsal hippocampus..... 207

Figure 5-5: Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septum (A) and dorsal hippocampal (B) infusion sites. 209

Figure 5-6: Bury time (LOG; s) in the shock probe apparatus after infusions of muscimol (2.5 ng/0.4 µl) into the medial septum, physostigmine (5 µg/1 µl) into the dorsal hippocampus, or combined infusions................ 211

List of abbreviations

5-HT	5-hydroxytryptamine (serotonin)
8-OH-DPAT	8-hydroxy-2-(n-dipropylamino)-tetraline hydrobromide
α	alpha
ACh	acetylcholine
AChE	acetylcholinesterase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AP	anterior posterior
BDA	biotinylated dextranamine
BZD	benzodiazepine
cm	centimetre
DH	dorsal hippocampus
DV	dorsal ventral
EC	entorhinal cortex
FFX	fimbria fornix
g	gram
GABA	gamma-aminobutyric acid
GAD	generalized anxiety disorder
h	hour
IL	Illinois
i.m.	intra-muscular

Inc.	incorporated
i.p.	intra-peritoneal
I.U.	international unit
kg	kilogram
LS	lateral septum
LSD	least significant difference
LTD	limited
µl	microlitre
µm	micrometre
M	mole
mA	milliampere
mg	milligram
min	minute
ML	medial lateral
mm	millimetre
MS	medial septum
MSDB	medial septum diagonal band
Musc	muscimol
NA	noradrenaline
NAAG	N-acetyl-aspartyl-glutamate
ng	nanogram
NIH	national institutes of health
nmol	nanomol

OCD	obsessive compulsive disorder
ON	Ontario
p	probability
PBS	phosphate-buffered saline
PD	panic disorder
PFA	paraformaldehyde
Physo	physostigmine
PTSD	post traumatic stress disorder
QC	Quebec
s.c.	subcutaneously
S.E.M.	standard error of the mean
TTX	tetrodotoxin
VA	Virginia
VH	ventral hippocampus

Chapter 1

Introduction

Background

Two emotions relevant to this dissertation are fear and anxiety. Although fear and anxiety have often been used interchangably they are in fact distinct (Andreatini et al., 2001). Generally, fear is a response to present danger whereas anxiety is a response to potential danger. More specifically, fear is an emergency defensive reaction enabling an organism to repond to immediate danger (Barlow, 2000). Anxiety, on the other hand, is focused largely on possible future threats, danger, or other negative stimuli (Barlow, 2000). Anxiety is accompanied by physical symptoms such as sweating, shaking or trembling, rapid heart beat, and shortness of breath. Although a normal emotional response, anxiety is abnormal if it occurs for no apparent reason, if it is prolonged, or if it is abnormally intense (Diagnostic and Statistical Manual of the American Psychiatric Association, 4th edition, 1994; DSM-IV). The DSM-IV classifies nine types of anxiety disorder: panic disorder (PD) without agoraphobia, PD with agoraphobia, agoraphobia, specific phobia, social anxiety disorder, generalised anxiety disorder (GAD), obsessive compulsive disorder (OCD), acute stress disorder, and post traumatic stress disorder (PTSD). Anxiety affects approximately 10% of the population (DSM IV).

Animal Models of Anxiety

Animal models of anxiety attempt to simulate some aspects of human anxiety disorders in order to study the neural mechanisms of anxiety and the mechanisms of action of anxiolytic compounds (Treit, 1994; Mineka, 1985; Marks, 1987). There are three important criteria for animal models of anxiety: correlation, isomorphism, and homology. A correlational model is selectively sensitive to clinically effective anxiolytic compounds. Therefore, correlational models have been used in prescreening novel anxiolytic compounds. An isomorphic model is a model in which the response of the animal to the anxiety provoking situation is similar to a human anxiety response. Finally, an animal model is homologous if the condition that produces anxiety in the animal would also result in anxiety in humans (Treit et al., 2003). One general assumption for animal models of anxiety is that a real or anticipated aversive stimulus should produce a fear reaction and this fear reaction should be decreased by anxiolytic agents. Animal models of anxiety can be divided into two categories. One category involves an animal's response to non-painful stressors, whereas the second category involves an animal's response to a painful stressor. An example of the former is the elevated plus-maze, whereas an example of the latter is the shock-probe burying test (see Fig. 1.1 and 1.2 respectively). Both the elevated plus-maze test and the shock-probe burying test, to varying degrees, meet some of the criteria of correlation, isomorphism, and homology (Treit et al., 2003).

The elevated plus-maze

The elevated plus-maze is composed of two open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm) each with an open roof, arranged such that the two arms of each type are opposite each other (see Fig. 1). The arms of the maze are elevated to a height of 50 cm above the floor. Rats placed in the elevated plus-maze typically avoid the open arms of the maze and spend most of their time in the two enclosed arms (Pellow et al., 1985). Confinement to the open arms of the maze produces significantly more anxiety related behavior (freezing, defecation, elevated plasma corticosterone concentrations) than confinement to the closed arms (Pellow et al., 1985). Anxiolytic drugs such as diazepam increase open-arm exploration in the plus-maze (Pellow, 1986; Pellow et al., 1985; Pellow and File, 1986; Treit et al., 1993b). Conversely, anxiogenic drugs such as yohimbine, decrease open-arm exploration (Johnston and File, 1989; Pellow, 1986; Pellow et al., 1985; Pellow and File, 1986). The test does not involve response learning or an explicit memory requirement, factors that can complicate the interpretation of drug effects in other animal models of anxiety (e.g., the conflict test; Treit, 1985).

The shock-probe burying test

The shock-probe burying test consists of a 40 (length) x 30 (width) x 40 (height) cm chamber that is made of transparent plexiglass and has an open roof. The floor of the chamber is covered evenly with a 5 cm layer of bedding material and an electrified probe protrudes in from one of the chamber walls (see Fig. 2). In this test, rats shocked from the stationary, electrified probe push bedding material from the floor of the experimental chamber toward or over the probe, with rapid alternating movements of the forepaws (i.e., "burying behavior"), while avoiding further contacts with the probe (Pinel and Treit, 1978; Treit et al., 1981; Treit et al., 1994). A reduction in the duration of probe burying, in the absence of a decrease in general activity, is used as the primary index of anxiety reduction in this test (Treit et al., 1981). Standard anxiolytic drugs such as diazepam decrease shock-probe burying (Treit et al., 1981; Tsuda et al., 1988; De Boer et al., 1990; Treit et al., 1993b; Treit et al., 1994), whereas anxiogenic drugs such as yohimbine increase shock-probe burying (Tsuda et al., 1988; Treit, 1990). The suppression of burying by benzodiazepine anxiolytics is not secondary to behavioral sedation (Treit and Fundytus, 1988;

Rohmer et al., 1990), associative learning deficits (Blampied and Kirk, 1983), or analgesia (Treit, 1985), and can be reversed by benzodiazepine antagonists such as flumazenil (Treit, 1987). An increase in shock-probe contacts in the absence of a decrease in general activity can also be used as an index of anxiety reduction in this test. For instance, the anxiolytic agents midazolam, pentobarbital, chlordiazepoxide, and buspirone all have been show to increase the number of probe contacts (Treit and Fundytus, 1988; Treit, 1990). Moreover, probe-shocked rats show increased plasma corticosteroid and adrenaline levels. These increases in plasma corticosteroid and adrenaline levels can be prevented by anxiolytic drugs (de Boer et al., 1990).

Animal models of anxiety have been used to study the neuroanatomical and neurochemical correlates of anxiety reactions in rodents. For instance, fluctuations in serotonergic, noradrenergic, GABAergic, and cholinergic levels have all been shown to influence anxiety reactions (e.g. Giovannini et al., 2001; Hajos-Korcsok et al., 2003; Hegarty et al., 1995). Regarding brain structures, limbic structures (e.g., amygdala, hippocampus, septum) in particular have been widely implicated in fear and anxiety. The role of the amygdala in anxiety has been extensively studied, but structures such as the septum and the hippocampus have also been shown to be important in anxiety regulation. These structures may regulate anxiety independently or through an interaction with other structures. For instance it is possible that the septum and hippocampus may interact in their modulation of anxiety. The septum and the hippocampus as well as the GABAergic and cholinergic systems will be the focus of subsequent sections.

The septum, hippocampus, and anxiety

Extensive evidence from lesion and drug studies have implicated the septum and to a lesser extent the hippocampus in fear and anxiety. Ablation of the septum reduces rats' anxiety-related behaviors in several animal models of anxiety. For example, electrolytic or excitotoxic lesions of the septum produced anxiolytic-like effects in the plus-maze and shock-probe burying tests (Pesold and Treit, 1992; Treit and Menard, 1997; Treit et al., 1993a). These anxiolytic effects occurred in the absence of hyperirritability (septal rage; Harrell and Balagura, 1975). Hippocampal lesions can also produce anxiolytic-like effects in animal models. For instance, hippocampal lesions impaired passive avoidance of the probe in the shock-probe burying test (Treit and Menard, 1997), increased open arm exploration in the elevated plus-maze test (Bannerman et al., 2002; Deacon et al., 2002; Kjelstrup et al., 2002), and reduced anxiety in the social interaction test (Bannerman et al., 2002). More specific evidence for the role of the septum and the hippocampus in anxiety modulation comes from studies using intracranial drug infusions. Infusions of GABAergic compounds in both structures have been shown to reduce anxiety. (Gonzalez et al., 1998; Menard and Treit, 2001). For instance, both hippocampal and septal infusions of the benzodiazepine-type anxiolytic, midazolam, an indirect $GABA_A$ agonist, increased open arm exploration in the plus-maze (Menard and Treit, 2001; Pesold and Treit, 1996). In addition, intra-septal infusions of midazolam decreased burying behavior in the shock-probe test (Pesold and Treit, 1994, 1996). Similarly, infusions of the direct $GABA_A$ agonist muscimol in the lateral septum (LS) decreased anxiety in the Vogel conflict test (Drugan, 1986). There are some data that suggest that infusions of cholinergic compounds in the septum or the hippocampus also modulate anxiety. Infusions of the nicotinic agonist nicotine in the LS increased anxiety (Ouagazzal et al., 1999), while infusions of both muscarinic and nicotinic antagonists in the hippocampus increased anxiety in a variety of tests (File et al., 1998; Hess and Blozovski, 1987; Smythe et al., 1998).

Septum and Hippocampus: Anatomy and Neurochemistry

Neurochemistry of the septum.

The septum can be divided into medial, lateral, and posterior regions (Jakab and Leranth, 1995). The LS is mainly composed of GABAergic neurons, whereas the medial septum-diagonal band (MSDB) contains both cholinergic and GABAergic neurons. Both types of neurons of the MSDB have cholinergic as well as GABAergic receptors (Bialowas and Frotscher, 1987). In addition, they possess receptors for other neurotransmitters, such as glutamate (Leranth and Frotscher, 1989). The proportion of medial septal neurons which are either cholinergic or GABAergic is not 100%, and the remaining neurons contain neuropeptides. The neuropeptides that are the most common are galanin and N-acetyl-aspartyl-glutamate (NAAG; Forloni et al., 1987; Melander et al., 1985; Senut et al., 1989). Some neuropeptides are colocalized with the cholinergic or GABAergic neurons. Galanin, for instance, is colocalized with cholinergic neurons and parvalbumin with GABAergic neurons (Melander et al., 1985).

Neurochemistry of the hippocampus.

Unlike the septum, the principal cells of the hippocampus are glutamatergic neurons. These glutamatergic neurons are comprised of both the granule cells of the dentate gyrus and the pyramidal cells of the hippocampus proper. The glutamatergic cells comprise 90% of hippocampal neurons. The remaining 10 % of hippocampal neurons are composed of GABAergic interneurons (Freund and Buzsaki, 1996). The pyramidal cells contain glutamatergic, cholinergic, GABAergic, serotoninergic, and noradrenergic receptors. The GABAergic interneurons have receptors for GABA, acetylcholine (ACh), noradrenaline (NA), and serotonin (Vizi and Kiss, 1998).

Septal and hippocampal connections.

The septum has a number of afferent and efferent connections that may mediate its role in fear and anxiety (see Figs. 3 and 4). The afferent and efferent connections of the LS and MSDB share some similarities, but some anatomical differences also exist. In general terms, the MSDB can be viewed as the relay center, transmitting ascending information from lower to higher brain regions. Conversely, the LS mediates descending information to brain stem areas (Leranth and Vertes, 2000). However, this view is oversimplified since the LS is also widely innervated by diencephalic sources (Jakab and Leranth, 1995). The MSDB and LS are reciprocally connected through GABAergic pathways (Leranth et al., 1992). Both the MSDB and the LS receive serotonergic, noradrenergic, and cholinergic inputs from brainstem areas. Specifically, the septum receives a serotonergic input from the raphé nuclei, a noradrenergic input from the locus coeruleus, and a cholinergic input from the tegmental nuclei (Leranth and Frotscher, 1989). The MSDB and LS also receive a glutamatergic projection from the hypothalamus. This glutamatergic pathway projects to cholinergic neurons in the MSDB and GABAergic neurons in the LS (Leranth and Vertes, 2000). The amygdala projects to the LS, but not the MSDB (Jakab and Leranth, 1995). The pathway from the amygdala to the LS is believed to be composed of neuropeptide Y-containing fibers (Allen et al., 1984). The hippocampus projects to the septum via both a GABAergic and glutamatergic pathway. Neurons in the MS receive GABAergic afferents from the hippocampus (Leranth et al., 1992), while the glutamatergic pathway projects to the LS. The GABAergic projection originates from non-pyramidal cells in the stratum oriens of the CA1-CA3 region and innervates cholinergic and non-cholinergic neurons (Amaral and Witter, 1995). The glutamatergic pathway projects from pyramidal cells and terminates on GABAergic neurons of the LS (Jakab and Leranth, 1990). In addition to the glutamatergic pathway from the hippocampus, the LS also receives a glutamatergic projection from the entorhinal cortex (EC; Stevens and Cotman, 1989).

The septum contains several efferent pathways. Both the MSDB and LS project to glutamatergic neurons of the hypothalamus via a GABAergic projection (Jakab and Leranth, 1995). In addition, both areas of the septum project to the amygdala. The MSDB-amygdala pathway is cholinergic in nature, while the LS-amygdala pathway is GABAergic (Jakab and Leranth, 1995). Furthermore, the MSDB, but not the LS, projects to the EC and the hippocampus.

Afferent pathways of the hippocampus.

The MS sends a cholinergic projection to the EC and is connected to the hippocampus through cholinergic and GABAergic projections. In the septo-hippocampal pathway, cholinergic fibers constitute between one half and two thirds of all septo-hippocampal projections, whereas the remaining non-cholinergic septo-hippocampal neurons are GABA-ergic (Freund and Antal, 1988; Kiss et al., 1990; Nauman et al., 1992; Wainer et al., 1985). These septal fibers reach the hippocampus via the fimbria and the dorsal fornix and terminate mainly in the dentate gyrus and subfields of Ammon's horn (Freund and Antal, 1988). The cholinergic neurons project to the hippocampal pyramidal cells, the dentate granule cells and the inhibitory interneurons (Lewis et al., 1967). The GABAergic neurons, on the other hand, project primarily to the inhibitory interneurons (Babb et al., 1988). The hippocampus is innervated by another major pathway, the glutamatergic perforant pathway, that originates from the entorhinal cortex. In addition, the hippocampus receives serotonergic and noradrenergic inputs from the raphé nuclei and the locus coeruleus respectively (Vizi and Kiss, 1998). Whereas the medial raphé nucleus projects mainly to the dorsal hippocampus the dorsal raphé nucleus projects mainly to the ventral hippocampus (Azmitia and Segal 1978). Another anatomical distinction between the ventral and dorsal hippocampus is that the ventral, but not the dorsal, hippocampus receives projections from, and sends projections to, the basolateral nucleus of the amygdala (Pikkarainen et al., 1999).

Given these neuroanatomical and neurochemical dissociations, it seems possible that different regions of the septum or the hippocampus play different roles in their mediation of fear and anxiety. In support of this concept, infusions of midazolam in the lateral, but not the medial, septum reduced anxiety in the plus-maze and shock-probe tests (Pesold and Treit, 1996). Additionally, lateral and medial septal lesions had different results on behavioral suppression (Feldon et al., 1982). Furthermore, stimulation of the lateral, but not the medial, septum inhibited aggressive behavior (Brayley and Albert, 1977). Regarding the hippocampus, a distinction was observed following the infusion of serotonergic compounds. Infusions of the serotonin (5-HT) 1_A agonist 8-OH DPAT in the ventral hippocampus had no effect on anxiety measures in the plus-maze test or social interaction (File and Gonzalez, 1996; Hogg et al., 1994). Conversely, the same dose of 8-OH DPAT, when infused in the dorsal hippocampus, induced a significant anxiogenic effect as measured in social interaction and plus-maze (File et al., 1996).

The GABAergic and Cholinergic Systems and Anxiety

As mentioned, stimulating GABAergic receptors in the septum or hippocampus decreases rats' fear reactions (Gonzalez et al., 1998; Menard and Treit, 2001; Pesold and Treit, 1996). It is generally believed that anxiety can result from changes in GABAergic transmission (DSM IV). Decreased GABAergic activity results in arousal, anxiety, restlessness, insomnia, and exaggerated activity (Nutt and Malizia, 2001). Although both $GABA_A$ and $GABA_B$ receptors are involved in the anxiogenic effect (Quintero et al., 1985; Shephard et al., 1992), $GABA_A$ receptors play a more prominant role (Cugurra, 1992) and are the target of benzodiazepines (BDZs), a class of well known anxiolytics. When GABA binds to the $GABA_A$ receptor, it produces an increase in the permeability of the chloride ion channel, which hyperpolarizes the post-synaptic neuron. Benzodiazepines bind to the $GABA_A$ receptor at a specific binding site that is different from the site where GABA binds. Benzodiazepines increase the binding of GABA to the receptor and increase the inhibitory actions of GABA (Tallman, 1985). More specifically, BDZs increase the frequency of the opening of the chloride channels (Twyman et al., 1989). They lower the concentration of GABA required to open the channel and thus enhance the effectiveness of GABA (Barnard et al., 1998). Clinically, BDZs are highly effective anxiolytics. Imaging studies indicate that patients suffering from some anxiety disorders exhibit a decrease in BDZ binding. This suggests that some anxiety disorders may be due to defective neuroinhibitory processes (Malizia et al., 1998; Malizia, 1999; Tiihonen et al., 1997). Further clinical evidence for the role of GABA in anxiety comes from patients that exhibit a deficit in $GABA_A$ receptor density. These patients are more likely to suffer from panic attacks and GAD (Schlegel, et al., 1994; Kaschka, et al., 1995; Malizia, et al., 1998).

Experimentally, an impairment of $GABA_A$ receptor function in an animal model is sufficient to induce a state of anxiety (Crestani et al., 1999). Conversely, a facilitation of $GABA_A$ receptor function attenuates animals' responses to fear-provoking stimuli. For instance, systemic infusions of BZDs decrease anxiety in a variety of animal models of anxiety, including the light-dark, social interaction, plus-maze, and shock-probe burying tests (Crawley, 1981; Guy and Gardner, 1985; Treit et al., 1993b). Benzodiazepine receptors are particularly abundant in limbic structures (Niehoff and Kuhar, 1983; Young and Kuhar, 1980), and studies using intracranial drug infusions suggest that limbic structures may modulate the anxiolytic effect induced by increased GABAergic stimulation (e.g. Gonzalez et al., 1998; Kataoka et al., 1982; Menard and Treit, 1999; Nazar et al., 1999; Pesold and Treit, 1994, 1995, 1996).

Whereas the role of the GABAergic system in anxiety modulation has been extensively studied, the role of the cholinergic system in anxiety modulation has received limited study. However it has been shown that like increased GABAergic function, increased cholinergic function can result in decreased anxiety levels (ie. Brioni et al., 1993; Rodgers and Cole, 1995; Smythe et al., 1998). Interestingly, Alzheimer's disease is associated with decreased cholinergic activity and roughly 33% of Alzheimer's patients suffer from anxiety disorders (Weiner et al., 1997). More importantly, treatment with acetylcholinesterase (AChE) inhibitors decreased anxiety in Alzheimer's patients (Weiner et al., 1997). In addition, studies have shown that nicotine can result in lower anxiety levels (Pomerleau et al., 1984; Jarvik et al., 1989).

Experimental evidence for the role of ACh in anxiety comes from animal models of anxiety. Systemic administration of cholinergic compounds are known to modulate anxiety in both rats and mice. Systemic injections of muscarinic antagonists increased anxiety in the plus-maze and black-white box (Rodgers and Cole, 1995; Smythe et al., 1998), whereas systemic injections of nicotinic agonists in rats or mice decreased anxiety in the elevated plus maze, social interaction, and contextual fear conditioning (Brioni et al., 1993; Decker et al., 1994; Irvine et al., 1999; Szyndler et al., 2001). Furthermore, systemic injections of the AChE inhibitor physostigmine decreased novelty-induced neophobia in rats (Sienkiewicz-Jarosz et al., 2000).

There is some limited evidence that suggests that the cholinergic and GABAergic systems can interact in anxiety modulation. Belotti et al., 1998 examined the combined effects of infusing a muscarinic antagonist (scopolamine) in the medial septum with a systemic injection of diazepam. Whereas a single injection did not affect anxiety, combined sub-effective doses of scopolamine and diazepam significantly alleviated anxiety as measured with the elevated plus-maze. This raises the possibility of an ACh-GABA interaction in anxiety regulation.

Thesis Rationale

Although much is known about the role of the septo-hippocampal system in anxiety regulation, several issues still need to be addressed. First, lesion work to this point has only examined the effects of permanent lesions while the effects of temporary lesions have not been explored. Since permanent lesions result in progressive degeneration of axons and cell bodies, and in a reorganization of brain tissue (Bishop, 1982; Eccles, 1976; Ramirez, 2001), this raises the possibility that the behavioral effects obtained with permanent lesions may be due to an effect on distant sites. Second, the role of hippocampal cholinergic systems in anxiety is still poorly understood. Although there is anatomical evidence of a difference between dorsal and ventral hippocampal cholinergic systems, the functional significance of these differences for anxiety regulation has not been systematically explored. Third, although the role of the GABAergic system in anxiety has received greater study, the effect of intra-cerebral infusions of directly acting GABA agonists has rarely been assessed. Finally, there is some suggestion that the septum and the hippocampus can interact in the modulation of anxiety, but only one prior study attempted to address this issue (Menard and Treit, 2001).

This thesis focuses on addressing these issues in an effort to enhance our understanding of the septo-hippocampal system in fear and anxiety. The roles played by various sub-regions of the septo-hippocampal system in anxiety modulation will be determined in the first set of experiments using temporary reversible lesions. The medial septum, fimbria fornix, dorsal and ventral hippocampus will be systematically lesioned while fear responses are measured. The roles played by the dorsal and ventral hippocampal cholinergic systems in anxiety modulation will be examined in the second set of experiments using intra-hippocampal infusions of the AChesterase inhibitor physostigmine. In the third set of experiments, activity in the medial or lateral septum will be suppressed using the directly acting GABAa agonist muscimol. In addition, the cholinergic and GABAergic systems of the hippocampus and septum will be stimulated simultaneously. The final set of experiments will explore the possibility of a septal-hippocampal interaction in greater detail by using sub-effective doses of physostigmine in the hippocampus and muscimol in the septum.

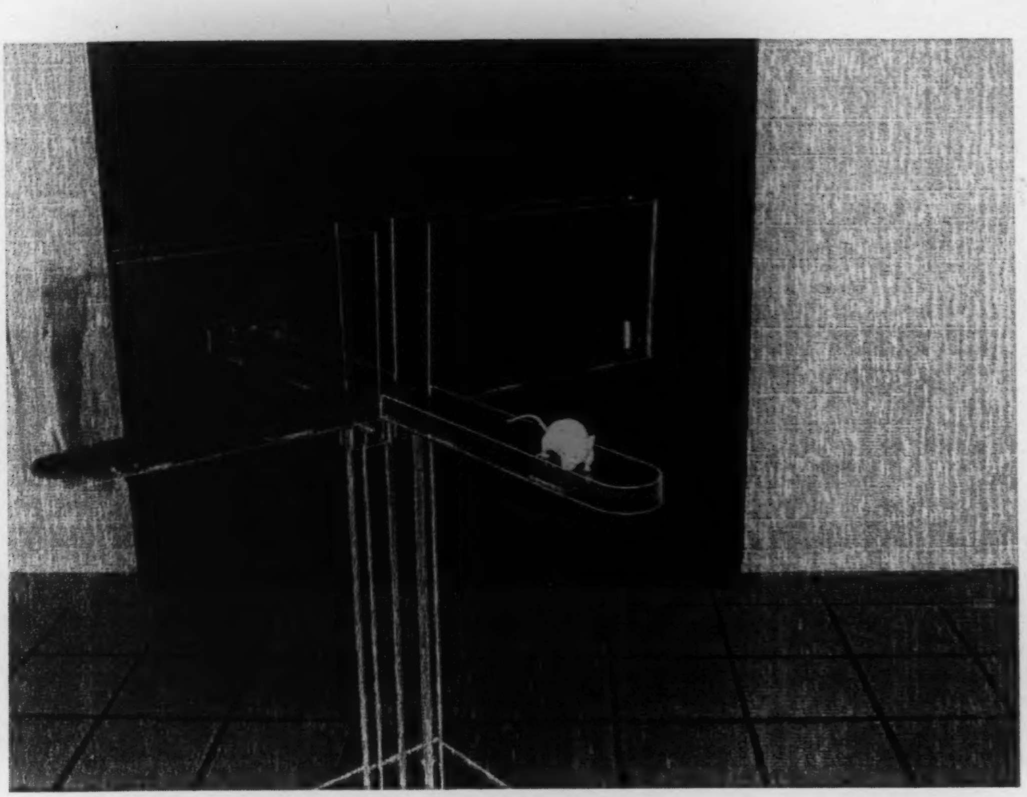


Figure 1-1. The elevated plus-maze.

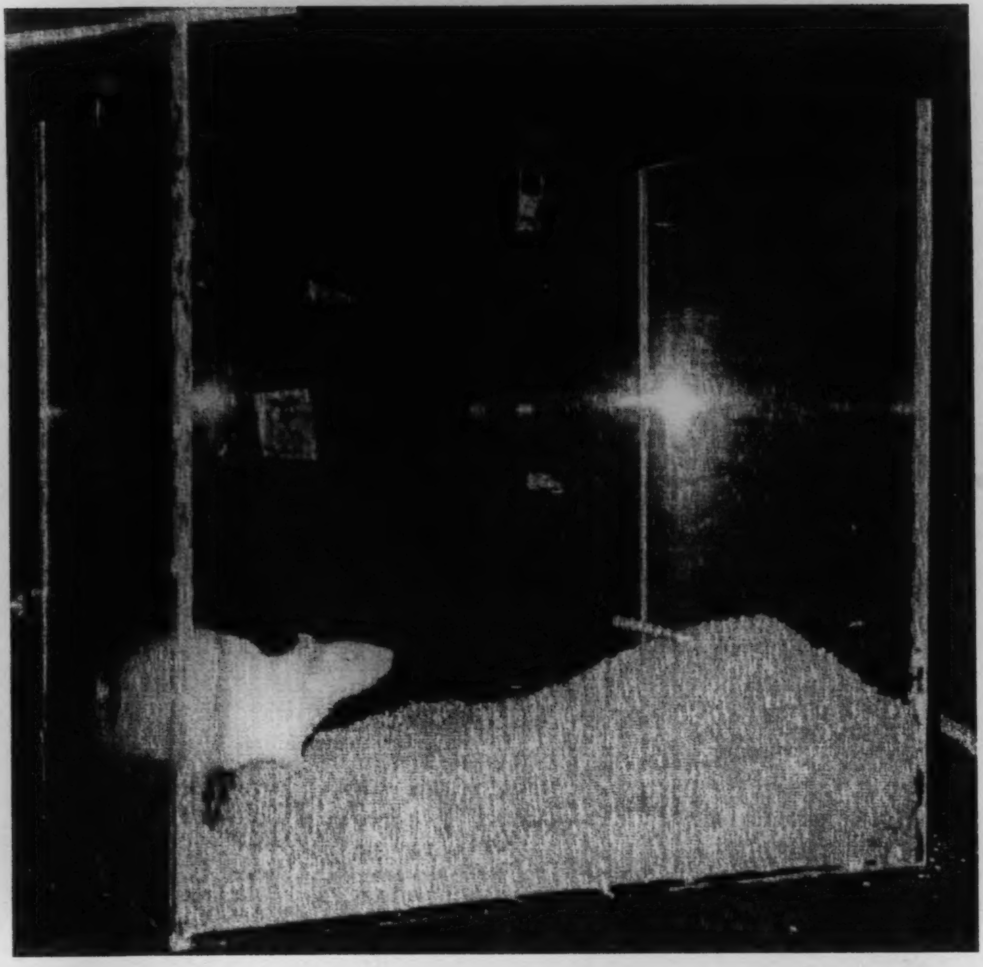


Figure 1-2. The shock-probe burying test.

Figure 1-3. Schematic representation of the anatomy of the septo-hippocampal system.

P: pyramidal cells, NP: non-pyramidal cells. ⬬ = ACh receptor

▬ = GABA receptor.

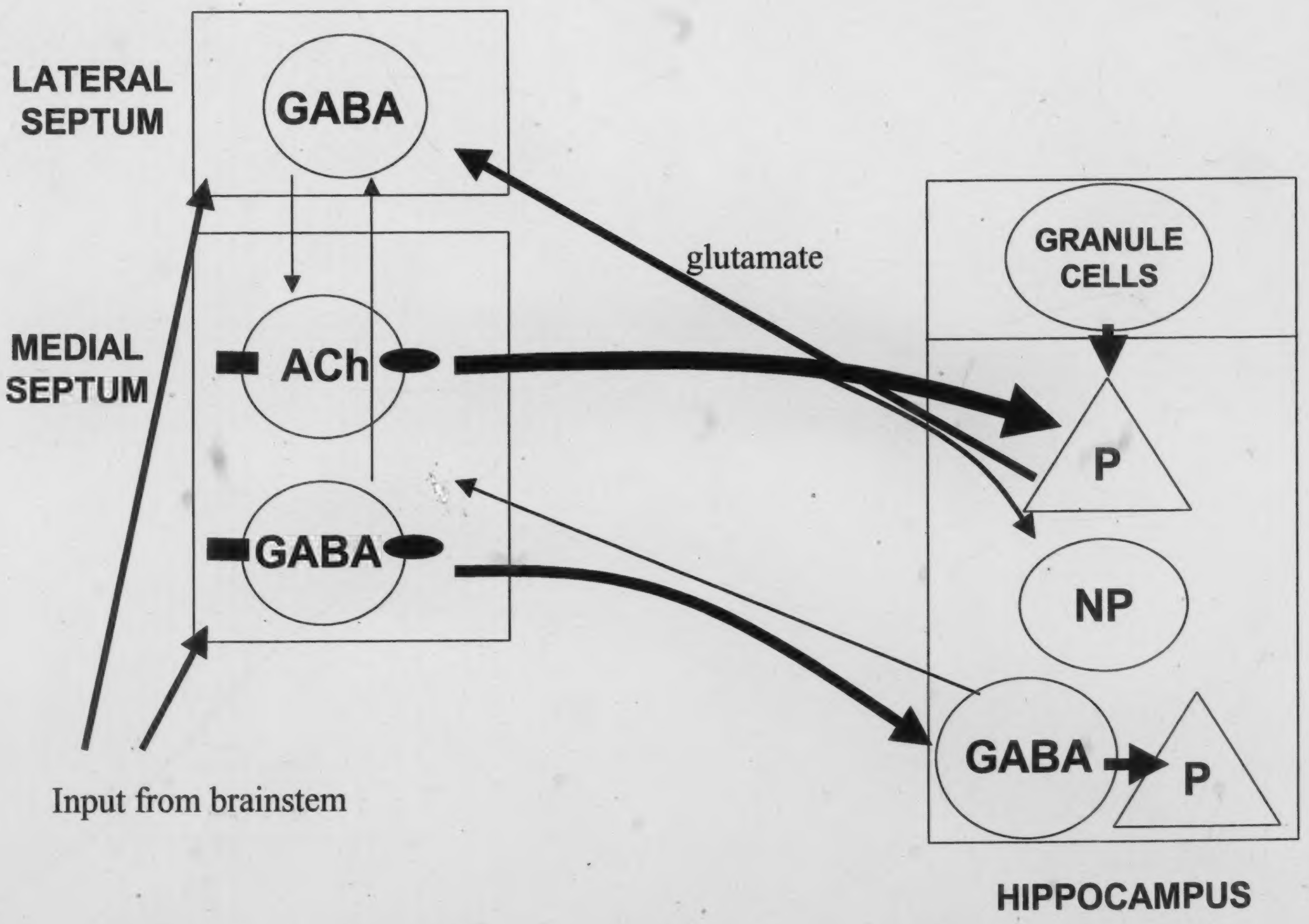
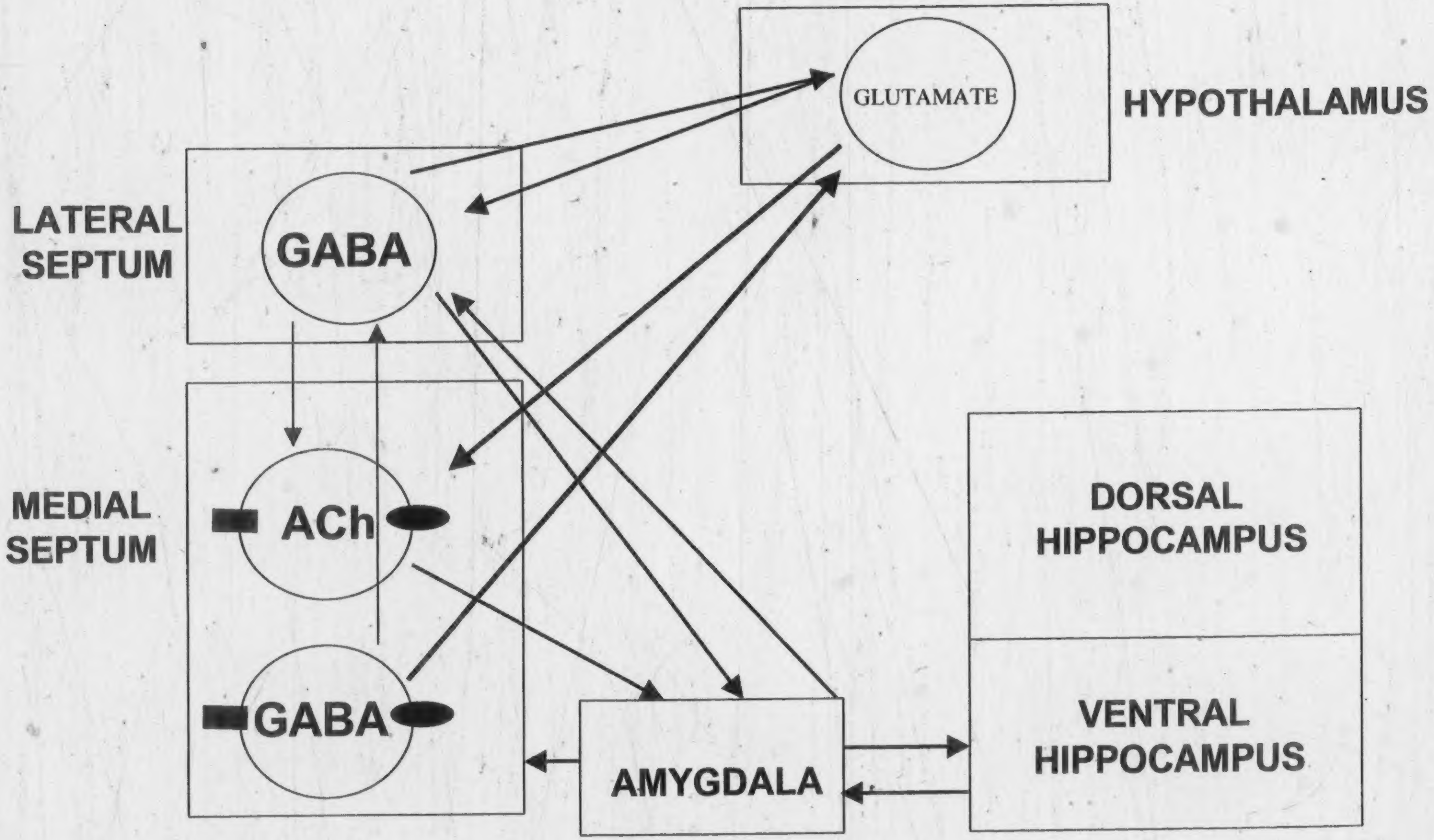

LATERAL SEPTUM
GABA
MEDIAL SEPTUM
ACh
GABA
glutamate
GRANULE CELLS
P
NP
GABA
P
Input from brainstem
HIPPOCAMPUS

Figure 1-4. Schematic representation of afferent and efferent connections of the septum and the hippocampus.


GLUTAMATE
HYPOTHALAMUS
LATERAL SEPTUM
GABA
MEDIAL SEPTUM
ACh
GABA
DORSAL HIPPOCAMPUS
VENTRAL HIPPOCAMPUS
AMYGDALA

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Chapter 2

Anxiety is functionally segregated within the septo-hippocampal system

A version of this chapter has been submitted for publication to *Brain Research*.

Introduction

Anatomically, the septum and the hippocampus are extensively connected. The septum contains cholinergic and GABAergic cell bodies that project to the hippocampus via the fimbria fornix (Freund and Antal, 1988; Kiss et al., 1990; Naumann et al., 1992; Wainer et al., 1985). The hippocampus, in turn, projects to the septum via both a GABAergic and glutamatergic pathway (Amaral and Witter, 1995).

Functionally, extensive evidence has implicated the septum and the hippocampus in fear and anxiety. For example, we have repeatedly found that ablation of the septum reduces rats' untrained fear reactions, suggesting that the septum normally plays an excitatory role in the control of anxiety (for reviews see; Menard and Treit, 1999; Treit and Menard, 2000). Specifically, we found that electrolytic or excitotoxic lesions of the septum produced anxiolytic-like effects in the plus-maze and shock-probe burying tests, i.e., open-arm exploration was increased and burying behavior was decreased (Pesold and Treit, 1992; Menard and Treit, 1996a,b; Treit and Pesold, 1990; Treit et al., 1993a). In addition, dorsal hippocampal lesions impaired passive avoidance of the probe in the shock probe test but had no effect on burying behavior or open arm exploration (Treit and Menard, 1997).

Thus far, all prior lesion studies examining the role of the septo-hippocampal system in anxiety have used permanent electrolytic, excitotoxic, or knife cut lesions. One drawback of this method is that permanent lesions can result in progressive degeneration of axons and cell bodies, and in a reorganization of brain tissue (e.g. Eccles, 1976). In terms of the septo-hippocampal system, prior research has shown that fimbria fornix, hippocampal, and septal lesions result in retrograde and anterograde neuronal degeneration (e.g. Gage et al., 1986; Ginsberg et al., 1999; Halim and Swerdlow, 2000; Hefti, 1986; Kojima, 1991; Mellgren, 1973; Sofronview and Isacson, 1988; Tuszynski et al., 1990). Thus, previous behavioral effects obtained with permanent lesions of the septo-hippocampal system are difficult to attribute to functional inactivation at the lesion site. Therefore, we wanted to determine the effects of restricted, reversible lesions of the septo-hippocampal system on anxiety. Because we wanted to temporarily inactivate very specific neuronal regions, the sodium channel blocker tetrodotoxin (TTX) was used. Prior research showed that at the dose and volume used in the current study, we could selectively inactivate an area with a radius of 0.5 mm (Zhuravin and Bures, 1991).

Materials and Methods

Anxiety was assessed in the elevated plus-maze and the shock-probe burying tests. In the elevated plus-maze test, rats typically avoid the open arms of the maze and spend most of their time in the two enclosed arms (Pellow et al., 1985). In the shock-probe burying test, rats shocked from a stationary, electrified probe push bedding material from the floor of the experimental chamber toward the shock-probe (i.e., burying) while avoiding further contacts with the probe (Pinel & Treit, 1978; Treit et al., 1981; Treit et al., 1994). Anxiolytic drugs such as diazepam increase open-arm exploration in the plus-maze and decrease burying toward the shock-probe (De Boer et al., 1990; Pellow, 1986; Pellow et al., 1985; Pellow & File, 1986; Treit et al., 1981; Treit et al., 1993b; Treit et al., 1994; Tsuda et al., 1988). Conversely, anxiogenic drugs such as yohimbine, decrease open-arm exploration while increasing shock-probe burying (Johnston & File, 1989; Pellow, 1986; Pellow et al., 1985; Pellow & File, 1986; Treit, 1990; Tsuda et al., 1988).

Subjects

Male Sprague-Dawley rats (Ellerslie, Edmonton, Alberta, Canada), weighing 250-300g upon arrival, were used. Following surgery, rats were individually housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle (lights on at 07.00h), with food and water available ad libitum. Behavioral testing occurred between 09.00 and 19.00 h. Measures were taken to minimize pain and discomfort to the animals and all research was carried out in accordance with the NIH guidelines regarding the care and use of animals for experimental procedures.

Surgery and Histology

For all experiments, rats were given an oral administration of the analgesic acetaminophen (Tylenol 120 mg/1.5 ml; McNeil, Guelph, ON, Canada) followed one hour later by atropine sulfate (0.1 mg/0.2 ml, i.p.; Ormond Veterinary Supply LTD., Cambridge, ON, Canada) to reduce respiratory complications due to the anaesthetic. The rats were then anaesthetized with pentobarbital (Nembutal 50 mg/kg, i.p.; Abbott Laboratories, Toronto, ON, Canada), hydrated with saline (3 cc, s.c.), and given the antibiotic penicillin (Division of Vétoquinol N.-A Inc. Lavaltrie, QC, Canada, 15,000 I.U./0.05 cc, i.m.). In experiment 1, stereotaxic procedures were used to implant 40 rats with two 22 gauge stainless-steel guide cannulae (Plastics One, Inc. Roanoke, VA) aimed bilaterally 1 mm above the fimbria fornix (-1.1 mm anterior to bregma [AP], 2.7 mm ventral to dura [DV], +/- 0.7 mm lateral to the midline, angled at 22°), using flat skull coordinates; Paxinos and Watson, (1986). In Experiment 2, stereotaxic procedures were used to implant 40 rats with one 22 gauge guide cannula aimed 1 mm above the medial septum (0.5 mm AP, 4.9 mm DV). Similarly in Experiment 3, the same surgical procedures were used to implant 40 rats bilaterally 1 mm above the ventral hippocampus (-5.2 mm AP, 5.7 mm DV, +/- 5.6 mm lateral to the midline). In Experiment 4, 40 rats were implanted bilaterally with guide cannulae aimed 1.2 mm above the dorsal hippocampus (-4.2 mm AP, 2.0 mm DV, +/- 4.1 mm lateral to the midline). Cannulae were implanted 1-1.2 mm above the intended target areas since the injection needle protrudes 1-1.2 mm beyond the cannulae tips.

In Experiment 5, we bilaterally infused 20 rats with the anterograde tracer biotinylated dextranamine (0.5 µl BDA; Molecular Probes Inc., US), at a rate of 0.05 µl/1 min, into the dorsal hippocampus at the same coordinate as that selected for Experiment 4 (-4.2 mm AP, 3.2 mm DV, +/- 4.1 mm lateral to the midline; n = 10) or ventral hippocampus at the same coordinate as that selected for Experiment 3 (-5.2 mm AP, 6.7 mm DV, +/- 5.6 mm lateral to the midline; n = 10). BDA was dissolved in 0.1 M phosphate-buffered saline (PBS) to create a 10% BDA solution. Subsequently the BDA solution was infused through 26 gauge stainless steel internal cannulae. The cannulae were connected to a 10 µl constant rate Hamilton microsyringe with polyethylene tubing and the infusions were delivered using an infusion pump (Harvard Apparatus 22). The cannulae were left in place for an additional 5 minutes following the infusion in order to allow the BDA to diffuse away from the cannula tip. The volume of BDA and the location of the infusion sites were based on the volume and location of TTX infused in experiments 3 and 4, since we wanted to determine the anatomical projections of a similar area to that inactivated by

TTX in these two experiments.

Guide cannulae in Experiments 1-4 were attached to the skull with 4 jeweler's screws and cranioplastic cement (Lang Dental MFG. Co., Inc, IL). Dummy cannulae were inserted into the guide cannulae to keep the cannulae tracts clear. Immediately after surgery the rats were placed into a warm environment until they regained consciousness. Two days after surgery, each cannula was checked for obstructions, and betadine (Purdue Pharma, Pickering, ON, Canada) was applied to the surgical wound. Following behavioral testing, rats were sacrificed with an overdose of chloral hydrate (Fisher, Nepean, ON, Canada) and perfused intracardially with 0.9% saline followed by 10% formalin (Fisher, Nepean, ON, Canada). The brains were removed and placed in a 10% formalin solution. After at least 48 hours had elapsed, the brains were frozen with dry ice, sectioned (60 µm), mounted onto glass slides and stained with thionin (Sigma, Oakvile, ON, Canada). The location of the cannulae for each rat was examined microscopically by an observer who was "blind" to the behavioral results. The location of the cannulae tips were then transcribed onto the appropriate atlas plates. The behavioral data for animals with misplaced cannulae or necrosis of brain tissue at the site of implantation were discarded. Using these criteria we deleted the data from 14, seven, nine, and six rats in Experiments 1,2, 3, and 4 respectively. In addition, behavioral data could not be obtained for animals that failed to touch the probe, or for animals that could not be properly infused. Because of these procedural problems, we were unable to obtain data from one rat in Experiment 3 and six rats in Experiment 4 in the plus maze test. In addition, we could not obtain data from five, two, and five rats in the shock probe apparatus in Experiments 1, 3, and 4 respectively.

Infusion Procedures in Experiments 1-4

Rats in each experiment were randomly assigned to one of the following drug conditions: a control condition, infused with vehicle (0.5 μl saline, pH 7.4) or a drug condition, infused with 5 ng/0.5 μl TTX (Sigma, Oakville, Ontario, Canada). These two drug conditions were counterbalanced across tests. The dose of TTX was based on a previous study (Baldi et al., 1998). The tame, hand-held rats were given an infusion of TTX or saline bilaterally into the fimbria fornix (Experiment 1), unilaterally into the medial septum (Experiment 2), bilaterally into the ventral hippocampus (Experiment 3), or bilaterally into the dorsal hippocampus (Experiment 4) through a 26 gauge stainless steel internal cannula lowered 1.0 mm (Experiments 1-3) or 1.2 mm (Experiment 4) below the tip of the guide cannula. The internal cannula was connected to a 10 μl constant rate Hamilton microsyringe with polyethylene tubing and the infusions were delivered using an infusion pump (Harvard Apparatus 22). The internal cannula was left in place for one minute following the infusions in order to allow for diffusion.

Anterograde Tracing Protocol Experiment 5.

Three days after the administration of BDA in Experiment 5, animals were anesthetized as described above and perfused transcardially for 20 minutes with 0.1 M PBS, pH 7.4, followed by refrigerated 4% paraformaldehyde (PFA) in 0.1 M PB, pH 7.4. Subsequently, the brain was removed, postfixed overnight in a 4 % PFA containing 15 % sucrose solution, and cryoprotected in 0.1 M PBS containing 15 % sucrose, pH 7.4.

Brains were sectioned in 50 um sections as described previously and sections were incubated overnight in ExtrAvidin peroxidase conjugate (Sigma, Oakville, Ontario, Canada) at 1:1000 dilution in 0.3% Triton PBS (Fisher, Canada). Subsequently, sections were stained for identification of the tracer as described in previous experiments (Wylie et al., 1997) in order to reveal structures anterogradely labeled by the transported BDA. Disminobenzidine (Sigma, Oakville, Ontario, Canada) staining was performed in combination with neutral red. Finally, terminal fields and injection sites were microscopically examined.

Plus Maze

The behavioral procedures were the same as those used in previous experiments (e.g., Degroot and Treit, 2002; Menard and Treit, 2000, Pesold and Treit, 1996; Treit et al., 1993a,b). All behavior was recorded on video-tape so that it could be analyzed at a later date, and all testing commenced at least seven days after the completion of surgery. The wooden, plus-shaped apparatus was elevated to a height of 50 cm, and consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, each with an open roof. The maze was in the center of a quiet and dimly lit room. The rat's behavior was observed using a mirror that was suspended at an angle above the maze. Behavioral data were collected by a "blind" observer who quietly sat one meter behind one of the closed arms of the maze. Five minutes following their respective drug treatment, rats were placed individually in the center of the plus-maze, facing one of the closed arms. The observer measured (1) time spent in the open arms, (2) time spent in the closed arms, (3) number of entries into the open arms, and (4) number of closed arm entries during the five-minute test period. An entry was defined as all four paws in the arm. The maze was cleaned with distilled water after each rat was tested. For the purpose of analysis (Pellow et al., 1985; Pellow and File, 1986), open-arm activity was quantified as the amount of time that the rat spent in the open arms relative to the total amount of time spent in any arm (open/total x 100), and the number of entries into the open arms was quantified relative to the total number of entries into any arm (open/total x 100). The total number of arms entered as well as the total number of closed arms entered were used as indexes of general activity (for details see Pellow et al., 1985; Rodgers and Johnson, 1995).

Shock Probe

The behavioral testing procedures were the same as those used in previous experiments (e.g., Menard and Treit, 2000, Pesold and Treit, 1996; Treit et al., 1993a,b). All behavior was recorded on videotape for ensuing analysis. All testing occurred at least seven days post-surgery.

The shock-probe burying apparatus consisted of a 40 x 30 x 40 cm plexiglass chamber, evenly covered with approximately 5 cm of bedding material (odor-absorbent kitty litter). The shock-probe was inserted through a small hole on one wall of the chamber, 2 cm above the bedding material. The plexiglass shock-probe (6.5 cm long and 0.5 cm in diameter) was helically wrapped with two copper wires through which an electric current was administered. Current from the shock source (2 pole precision animal shocker, Model H13-15, Coulbourn Instruments) was set at 2 mA. Rats were habituated in pairs to the test chamber without the shock-probe, for 30 minutes on each of four consecutive days prior to the test day. On the test day, five minutes following their respective drug treatments, rats were individually placed in one corner of the testing chamber, facing away from the shock-probe. The probe was not electrified until the rat touched it with its snout or forepaws, at which point the rat received a brief, 2 mA shock. The 15-minute testing period began once the rat received its first shock and the probe remained electrified for the remainder of the testing period. Following the first shock, the duration of time each rat spent spraying bedding material toward or over the probe with its snout or forepaws (i.e. burying behavior) was measured, as was the total number of contact-induced shocks each rat received from the probe. An index of the rat's reactivity to each shock was scored according to the following four-point scale (Pesold and Treit, 1992): (1) flinch involving only head or forepaw, (2) whole body flinch, with or without slow ambulation away from the probe, (3) whole body flinch, and/or jumping, followed by immediate ambulation away from the probe, and (4) whole body flinch and jump (all four paws in the air), followed by immediate and rapid ambulation (i.e. running) to the opposite end of the chamber. A mean shock reactivity score was calculated for each rat by summing its shock reactivity scores and dividing by the total number of shocks it received. The total time that the rat spent immobile (e.g. resting on the floor) during the 15-minute testing period was used as an index of general activity. All behavioral measures were made by a "blind" observer who was watching the rat via a video monitor in a room adjacent to the testing room.

Statistical Analysis

Results are expressed as means and standard errors of the mean (S.E.M.). The data were analyzed using analysis of variance (ANOVA). An alpha level of 0.05 was used as the criterion for statistical significance. In order to correct for non-normality and heterogeneity of variance, the burying scores were transformed to their natural logarithms prior to ANOVA.

Results

Experiment 1

Plus-maze

Figure 2-1 shows the infusion sites in the fimbria fornix. Figure 2-2 indicates that TTX infusions into the fimbria fornix produced clear anxiolytic-like effects in the plus-maze. Specifically, rats infused bilaterally with 5 ng of TTX into the fimbria fornix showed a significantly greater percentage of open-arm entries [F(1,24) = 6.23, p = 0.02] and open-arm time [F(1,24) = 10.57; p = 0.003] than their vehicle-infused controls. There was no indication of non-specific changes in general activity, since neither the total number of arms entered [F(1,24) = 0.37; p = 0.55; see table 2-1] nor the closed arm entries [F(1,24) = 0.53; p = 0.48; see table 2-1] differed between groups.

Shock-probe

Bilateral infusions of TTX in the fimbria fornix also increased the number of shocks taken by the rats (figure 2-3). Rats infused with TTX made significantly [F(1,19) = 5.63, p = 0.03] more contacts with the electrified probe than their respective vehicle-infused controls. In contrast, TTX lesions of the fimbria fornix did not affect the duration of burying [F(1,19) = 0.03, p = 0.87]. The effect on probe contacts occurred in the absence of any significant changes in immobility [F(1,19) = 0.00, p = 0.98], or shock reactivity [F(1,19) = 0.004, p = 0.95; see table 1].

Taken together, these data suggest that the temporary neuronal inactivation of the fimbria fornix with a 5 ng dose of TTX produces behaviorally specific anxiolytic-like effects in both the plus-maze and shock-probe burying tests.

Experiment 2

Plus-maze

Figure 2-4 shows the location of the infusion sites in the medial septum. The infusion of TTX in the medial septum increased the percentage of open arm entries [$F(1,31) = 34.14$, $p < 0.001$] (figure 2-5, panel A) and open arm time [$F(1,31) = 29.58$, $p < 0.001$] (figure 2-5, panel B) compared to the vehicle infused control group. Rats infused with TTX entered the closed arms significantly less than vehicle infused animals [$F(1,31) = 14.23$, $p = 0.001$; see table 2-1], but it is difficult to attribute this difference to a reduction in activity. The total number of arms entered [$F(1,31) = 0.099$, $p = 0.76$; see table 2-1] did not differ significantly between the groups, and TTX infused animals made significantly more open arm entries [$F(1,31) = 10.09$, $p = 0.003$] than vehicle infused controls. If infusions of TTX reduced activity levels, then the total arm entries and open arms entered should also have been reduced. In our experiment, rats infused with TTX were more likely to enter the open arms and less likely to enter the closed arms than vehicle infused controls. Thus the decrease in closed arm entries was likely due to an anxiolytic effect, rather than an effect on general activity.

Shock-probe

Figure 2-6 shows that infusions of TTX in the medial septum greatly reduced burying behavior. Statistical analysis confirmed that TTX-infused rats buried the shock-probe at significantly lower levels than vehicle controls [$F(1,31) = 22.96$, $p < 0.001$]. Unlike infusions of TTX in the fimbria fornix, infusions in the medial septum did not affect the number of shocks received [$F(1,31) = 2.85$, $p = 0.10$]. The effect on burying was not confounded by between-group differences in general activity [$F(1,31) = 1.67$, $p = 0.21$; see table 2-1] or shock reactivity [$F(1,31) = 0.07$, $p = 0.80$; see table 2-1].

Experiment 3

Plus-maze

Figure 2-7 shows the infusion sites in the dorsal hippocampus. Infusions of TTX into the dorsal hippocampus did not produce anxiolytic-like effects in the plus-maze. Tetrodotoxin infusions had no effect on the percentage of open-arm entries [$F(1,26) = 1.14$, $p = 0.30$; see table 1] or open-arm time [$F(1,26) = 1.80$; $p = 0.19$; see table 2-1]. In addition, there was no effect on general activity since neither the total arm entries [$F(1,26) = 0.16$; $p = 0.70$; see table 2-1], nor the closed arm entries [$F(1,26) = 0.43$; $p = 0.52$; see table 2-1] differed between groups.

Shock-probe

Figure 2-8 indicates that dorsal hippocampal TTX infusions increased the number of shocks taken by the rats. Rats infused with TTX in the dorsal hippocampus made significantly [$F(1,27) = 7.1$, $p = 0.01$] more contacts with the probe than their respective vehicle-infused controls. However, like fimbria fornix lesions, there was no effect on burying time [$F(1,27) = 0.05$, $p = 0.82$]. The effect on contacts made with the electrified probe occurred in the absence of any significant changes in immobility [$F(1,27) = 0.09$, $p = 0.76$] or shock reactivity [$F(1,27) = 0.32$, $p = 0.58$;

see table 2-1]. Thus, infusions of TTX in the dorsal hippocampus impairs passive avoidance of the electrified probe in the burying test, but does not impair passive avoidance of the open arms in the plus-maze test.

Experiment 4

Plus-maze

Figure 2-9 shows the location of the infusion sites in the ventral hippocampus. Infusion of TTX in the ventral hippocampus increased the percentage of open arm entries [F(1,28) = 14.35, p = 0.001] (figure 2-10, panel A) and open arm time [F(1,28) = 9.83, p = 0.004] (figure 2-10, panel B) compared to the vehicle-infused control group. Rats infused with TTX also made significantly fewer closed arm entries than vehicle infused animals [F(1,28) = 9.91, p = 0.004; see table 2-1]. However, the total number of arms entered [F(1,28) = 0.12, p = 0.73; see table 2-1] did not differ significantly between the groups, and TTX-infused animals made significantly more open arm entries [F(1,28) = 8.56, p = 0.007] than vehicle-infused controls. Therefore, the effect on the number of closed arms entered likely resulted from a decrease in anxiety as opposed to decreased activity levels.

Shock-probe

Figure 2-11 shows that infusions of TTX in the ventral hippocampus substantially reduced burying behavior. Statistical analysis confirmed that TTX-infused rats buried the shock-probe at significantly lower levels than vehicle controls [F(1,27) = 11.55, p = 0.002]. However, infusions of TTX into the ventral hippocampus did not affect the number of shocks taken by the rats [F(1,27) = 0.47, p = 0.50]. Furthermore, the effect on burying activity was not confounded by between-group differences in general activity [F(1,27) = 2.62, p = 0.12; see table 2-1] or shock reactivity [F(1,27) = 0.13, p = 0.72; see table 2-1].

Experiment 5

Location of BDA injection sites.

The BDA could be seen through a brown BDA reaction product. The sites of injection of the BDA into the dorsal or ventral hippocampus were characterized by densely labeled neurons (Figures 2-12 and 2-13, panels A). Within the dorsal hippocampus, tracer was located in the CA1 region and the alveus. More sparse labeling could be observed in the CA2 region. Within the ventral hippocampus, tracer was mainly confined to the CA1 and CA3 regions, but some could also be found in the CA2 area.

Anterograde labeling in the amygdala.

Dense terminal labeling was observed in the amygdala [basolateral nucleus, central nucleus, basomedial nucleus when BDA was infused in the ventral, but not the dorsal hippocampus (Figures 2-12 and 2-13, panels B)].

General Discussion

Our study was the first to examine anxiolytic-like effects of reversible lesions along the septo-hippocampal axis. As stressed in the introduction, permanent lesions can result in anterograde and retrograde degeneration and a reorganization of neural tissue (Kolb and Whishaw, 1996). In our study we inactivated a small neuronal region (0.5 mm in radius; Zhuravin and Bures, 1991) for a limited period of time in an effort to avoid the neuronal changes associated with permanent lesions. In addition, our study was the first to examine the effects of ventral hippocampal and fimbria fornix lesions on behavior in the shock-probe test. Similar to previous studies using irreversible electrolytic or excitotoxic septal lesions (Menard and Treit, 1996a, 1996b; Pesold and treit, 1992; Treit and Pesold, 1990; Treit et al., 1993b) TTX lesions of the septum increased open-arm exploration in the plus-maze test and decreased burying behavior in the shock-probe test, while having no effect on passive avoidance of the shock probe. In addition, TTX lesions of the fimbria fornix and ventral hippocampus produced an anxiolytic effect in the plus- maze that was similar to that obtained with electrolytic lesions of the fimbria fornix (Decker et al., 1995) and excitotoxic lesions of the ventral hippocampus (Bannerman et al., 2000). Infusions of TTX in the dorsal hippocampus had an effect on anxiety that was comparable to the anxiolytic effect obtained with electrolytic lesions (Treit and Menard, 1997), but not excitotoxic lesions (Bannerman et al., 2000). Unlike the effect obtained with excitotoxic lesions, infusions of TTX in the dorsal hippocampus did not affect open arm exploration in the plus maze. These results were similar to results obtained by Kjelstrup and colleagues (2002), who showed that the ventral, but not the dorsal, hippocampus was involved in defensive and fear-related behavior.

It has been proposed, based on early lesion studies, that the septum and the hippocampus generally act in a unitary fashion in the modulation of fear and anxiety (Gray, 1982; Gray and McNaughton, 1983). Indeed, the remarkable correspondence between the effects on anxiety of septal and ventral hippocampal lesions in our study is consistent with this suggestion. Furthermore, TTX lesions of the pathway connecting the two structures also had a profound effect on anxiety. However, we also observed functional segregation within the septo-hippocampal system in our study. For instance, there was a pronounced difference between the effects on anxiety of dorsal and ventral hippocampal lesions. This difference would not be expected if the septo-hippocampal system acts as one entity in anxiety regulation. Instead we would predict that disrupting any part of this system should have a similar effect on all anxiety responses. On the contrary, as in previous experiments (Menard and Treit, 1998; Treit and Menard, 1997), we found that different parts of the septo-hippocampal system exert differential control over very specific anxiety reactions (e.g. open-arm avoidance versus shock-probe avoidance). The idea that one "system" might be composed of several sub-systems, each of which makes a distinct contribution to a general neuronal process, such as sensorimotor control, is not a novel concept. In the context of sensorimotor control, for example, adaptive function appears to be executed by a system of distributed, parallel processing units, each specialized for a specific aspect of the sensorimotor world (Zeki, 1993). Likewise, it seems reasonable to hypothesize that the control of anxiety might be executed, in part, by distinct, parallel processing units within the septo-hippocampal system, each specialized for a specific danger or threat (Treit and Menard, 1997).

Similar to the effect of dorsal (but not ventral) hippocampal lesions observed in the present study, lesions of the amygdala also impair passive avoidance of the probe, without having effects on burying behavior in the shock-probe test, or open-arm exploration in the plus-maze test (Treit and Menard, 1997). This raises the possibility that in spite of the specific and temporary nature of our lesions, the anxiolytic effect observed following TTX inactivation of the dorsal hippocampus may have been mediated through the amygdala. If the amygdala was involved in the effect obtained with dorsal hippocampal lesions, then we would have expected to find labeled terminal regions in the amygdala following injections of BDA in the dorsal hippocampus. Instead, we found dense labeling in the amygdala following injections of BDA in the ventral, but not the dorsal, hippocampus. The absence of an apparent pathway between the dorsal hippocampus and the amygdala supports previous research (Pikkarainen et al., 1999) and makes it unlikely that the amygdala contributed to the anxiolytic effect observed in the present studies. This also suggests that our TTX lesions did not exert their behavioral effects through an effect on distal structures as might be seen with permanent lesions.

In conclusion, our experiments suggest that anxiety is functionally segregated along the septo-hippocampal system. Septal and ventral hippocampal TTX lesions increased open arm exploration in the plus-maze, suppressed burying behavior in the shock probe apparatus, but had no effect on shock probe contacts. In contrast, fimbria fornix and dorsal hippocampal TTX lesions impaired avoidance of the probe, but did not affect burying behavior. Fimbria fornix, but not dorsal hippocampal TTX lesions increased open arm exploration in the plus maze. The reason for the functional segregation of behavior in the hippocampus remains unclear, but could be attributed to differences in connections with the septum.

Table 2-1. Mean (+/- S.E.M.) percentage open arm exploration, total arm entries, and closed arm entries, in the plus-maze task, and mean (+/- S.E.M.) activity and reactivity in the shock-probe burying test.

Plus-maze

	Fimbria Fornix		Medial Septum		Ventral Hippocampus		Dorsal Hippocampus	
	Saline (n = 10)	TTX (n = 16)	Saline (n = 17)	TTX (n = 16)	Saline (n = 14)	TTX (n = 16)	Saline (n = 16)	TTX (n = 12)
Total arm entries	8.40	9.31	10.65	11.19	13.86	14.38	11.56	12.17
	(1.22)	(0.92)	(1.14)	(1.29)	(1.07)	(1.06)	(1.00)	(1.16)
Closed arm entries	7.00	6.06	7.35	3.63	9.50	5.88	7.75	7.00
	(1.02)	(0.80)	(0.83)	(0.52)	(0.98)	(0.65)	(0.80)	(0.77)
Percentage open arm entries	16.40	33.38	28.65	66.75	30.79	58.06	33.50	40.92
	(3.66)	(4.84)	(4.89)	(4.26)	(5.97)	(4.26)	(4.68)	(5.03)
Percentage open arm time	5.60	22.44	19.18	60.31	24.82	52.81	20.31	30.83
	(1.82)	(3.91)	(5.05)	(5.66)	(6.56)	(6.07)	(3.66)	(7.67)

Shock-probe

	(n = 11)	(n = 10)	(n = 16)	(n = 17)	(n = 12)	(n =17)	(n = 14)	(n = 15)
Immobility (s)	102.27	103.60	27.56	76.88	34.92	105.94	130.21	150.13
	(51.96)	(39.31)	(12.60)	(35.07)	(19.18)	(34.16)	(48.67)	(43.11)
Shock reactivity	1.92	1.90	2.47	2.54	1.92	2.00	2.15	2.07
	(0.21)	(0.18)	(0.17)	(0.20)	(0.15)	(0.17)	(0.11)	(0.08)
Shock number	1.82	2.70	1.69	2.06	2.17	1.94	2.21	3.20
	(0.23)	(0.30)	(0.12)	(0.18)	(0.27)	(0.20)	(0.21)	(0.30)
Burying time (LOG)	2.05	2.34	3.84	0.83	4.29	1.85	3.06	2.87
	(0.55)	(0.55)	(0.46)	(0.42)	(0.27)	(0.57)	(0.57)	(0.59)

Figure 2-1. Schematic illustration of coronal sections of the rat brain showing the approximate location of fimbria fornix infusion sites. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from Paxinos and Watson (1986).

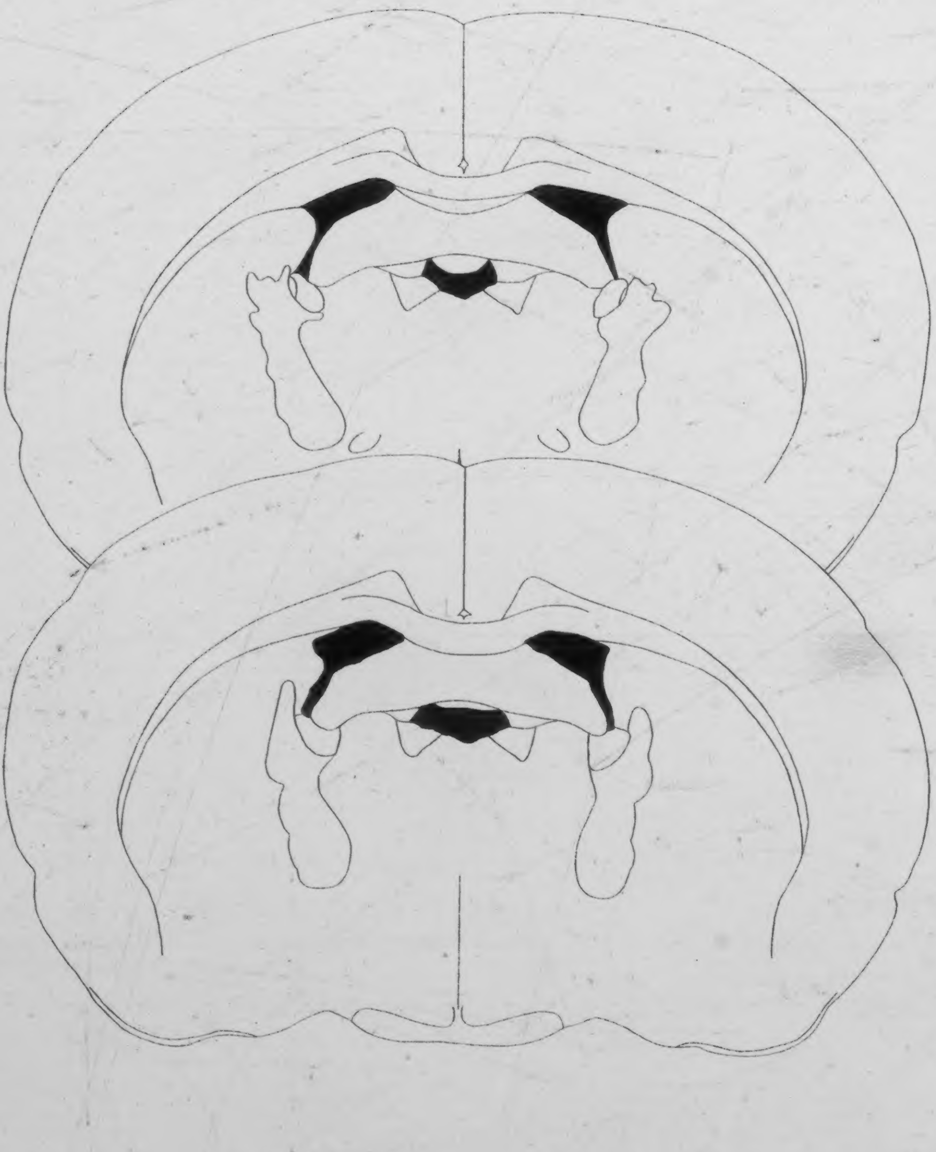


Figure 2-2. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of TTX (5 ng/0.5 μl) or vehicle (0.5 μl) into the fimbria fornix. *p < .05 compared with the vehicle control group.

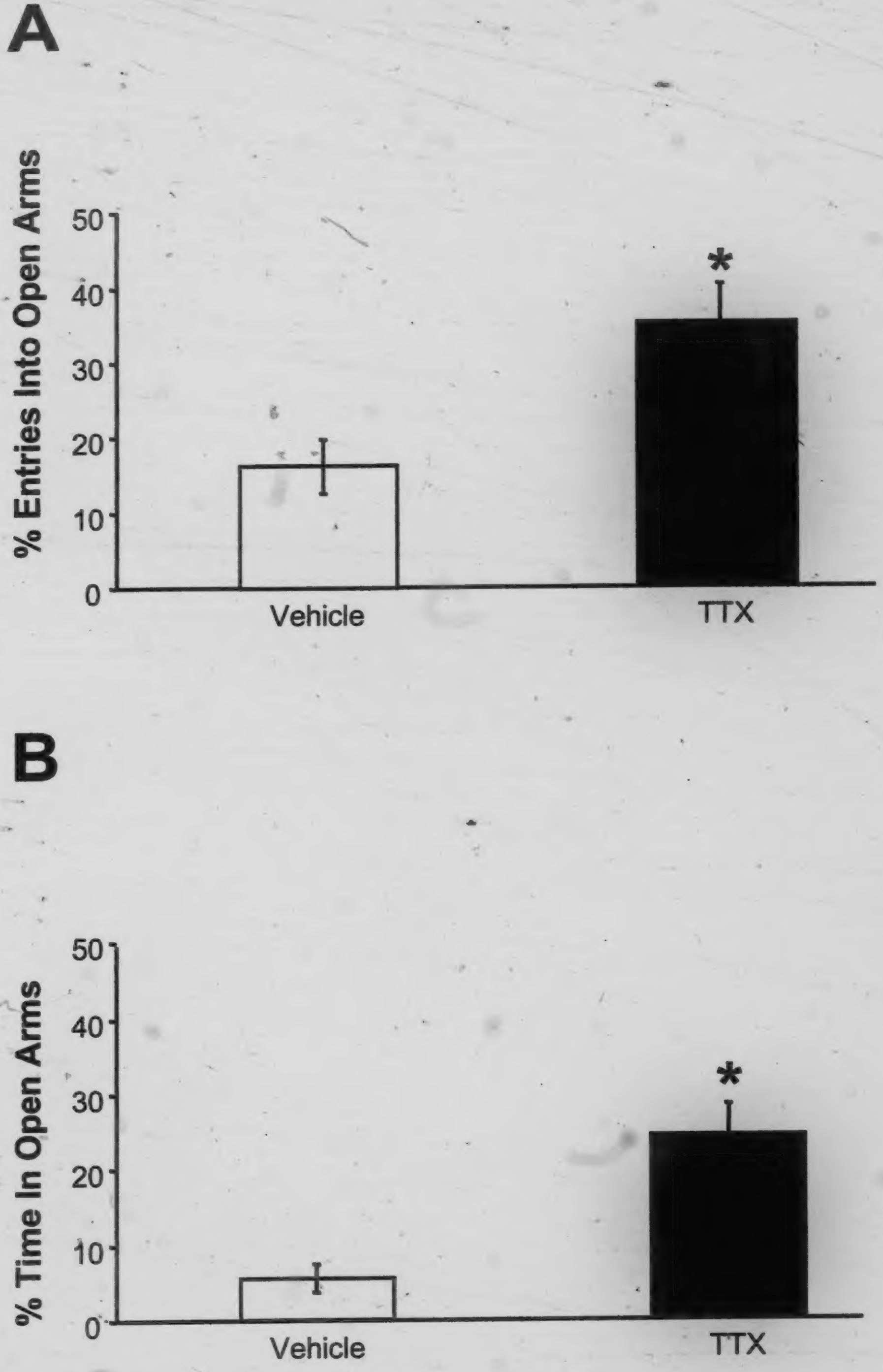

A
% Entries Into Open Arms
50
40
30
20
10
0
*
Vehicle
TTX
B
% Time In Open Arms
50
40
30
20
10
0
*
Vehicle
TTX

Figure 2-3. Mean (+/- S.E.M.) number of shocks in the shock probe apparatus after infusions of TTX (5 ng/0.5 µl) into the dorsal hippocampus. *$p \leq .05$ compared with the vehicle control group.

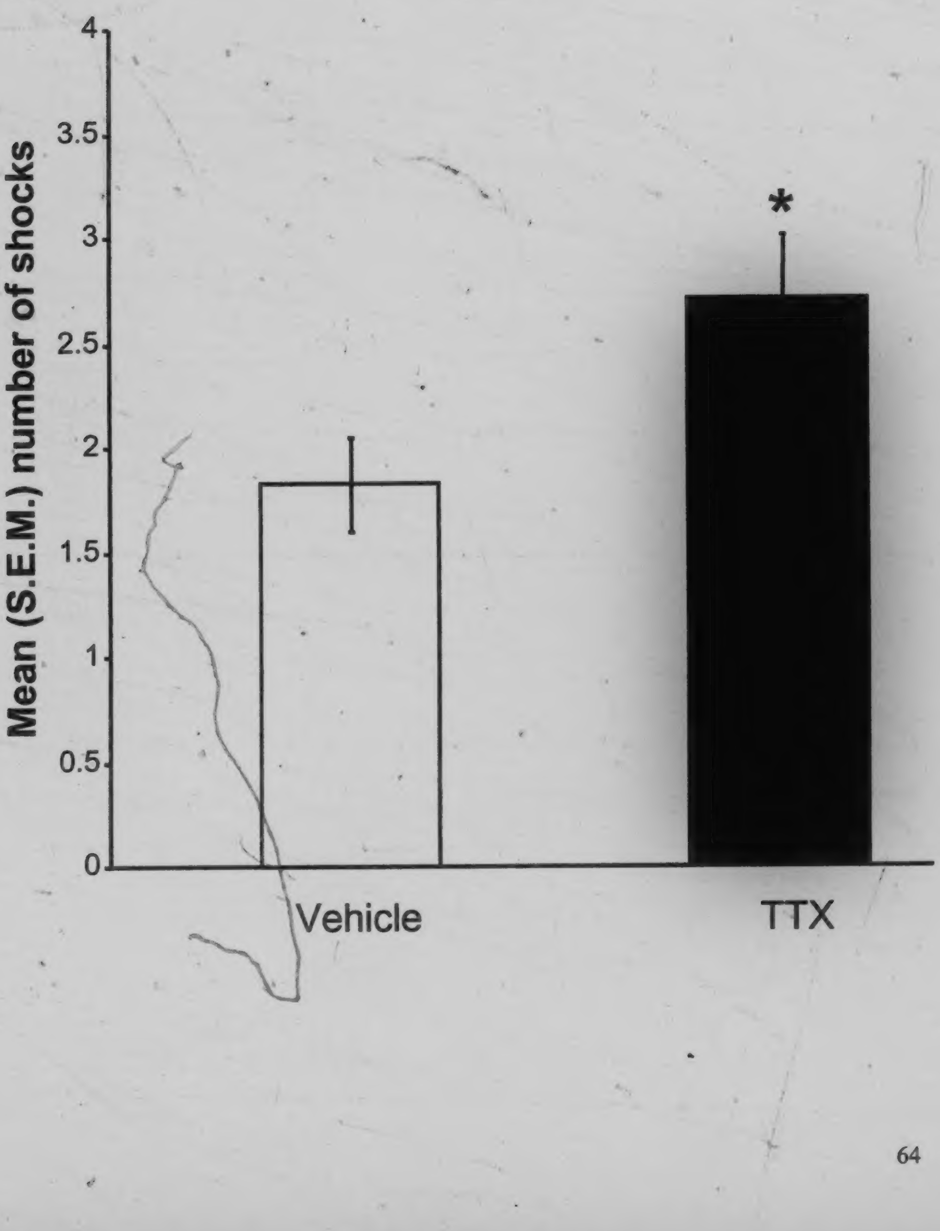

Mean (S.E.M.) number of shocks
4
3.5
3
2.5
2
1.5
1
0.5
0
Vehicle
TTX
*

Figure 2-4. Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septal infusion sites in Experiment 2. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from (Paxinos and Watson, 1986).

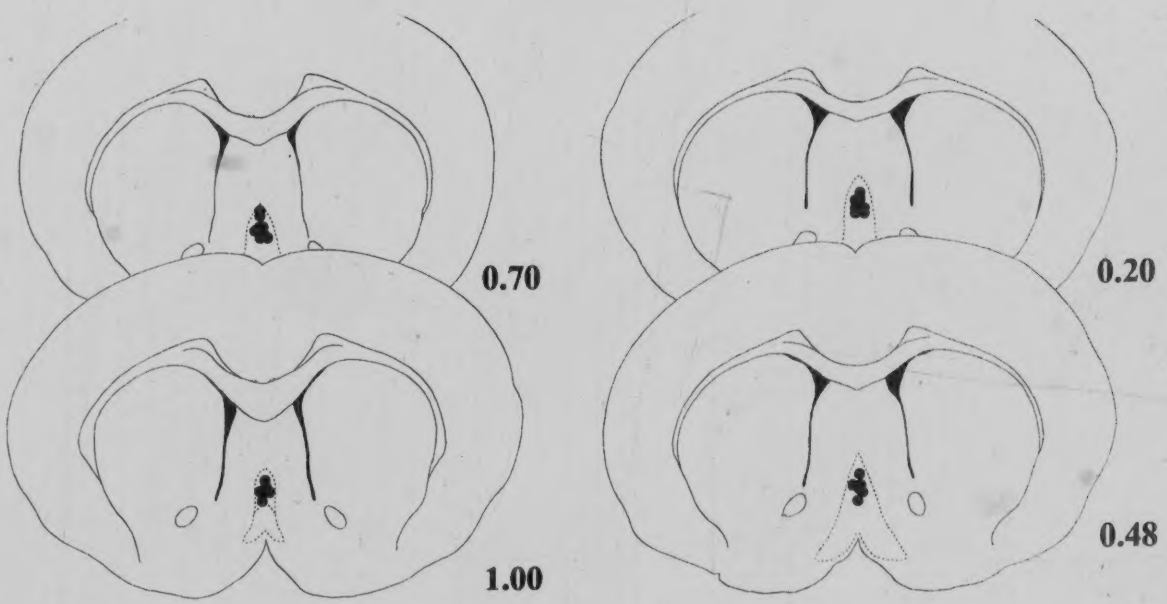
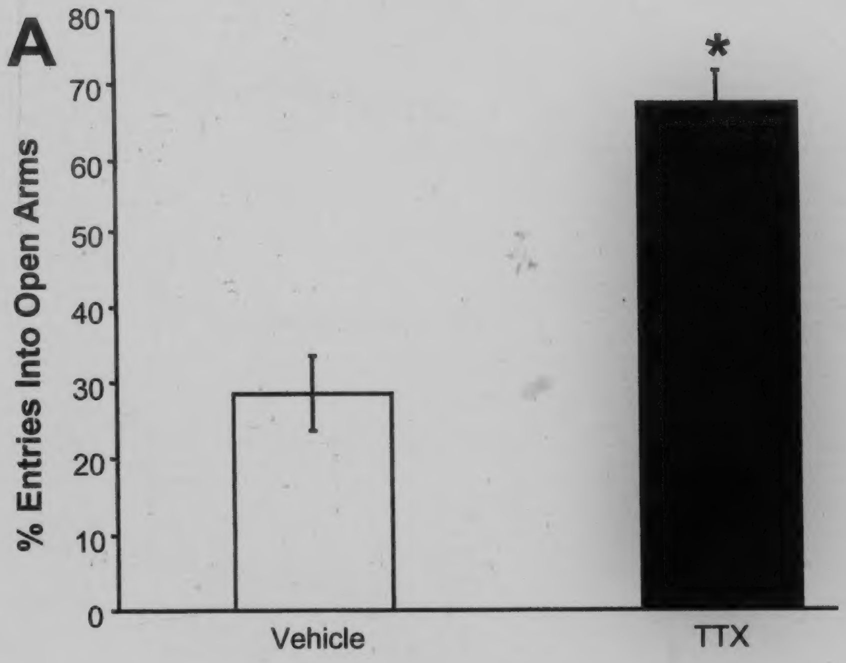

0.70
0.20
1.00
0.48

Figure 2-5. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of TTX (5 ng/0.5 μl) into the medial septum. *p < .05 compared with the vehicle control group.


A
80
70
60
50
40
30
20
10
0
% Entries Into Open Arms
*
Vehicle
TTX

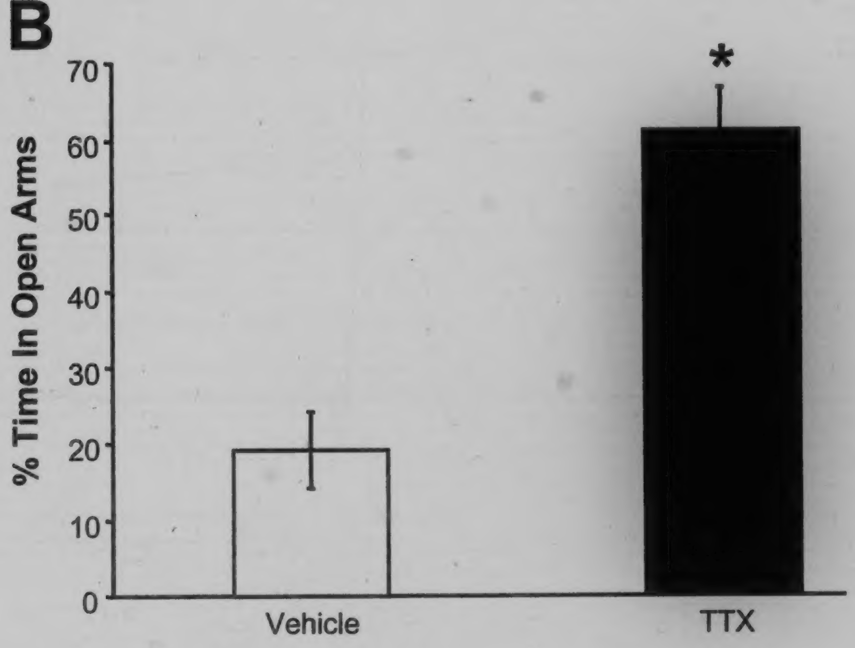

B
70
60
50
40
30
20
10
0
% Time In Open Arms
*
Vehicle
TTX

Figure 2-6. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of TTX (5 ng/0.5 µl) into the medial septum. *p < .05 compared with the vehicle control group.

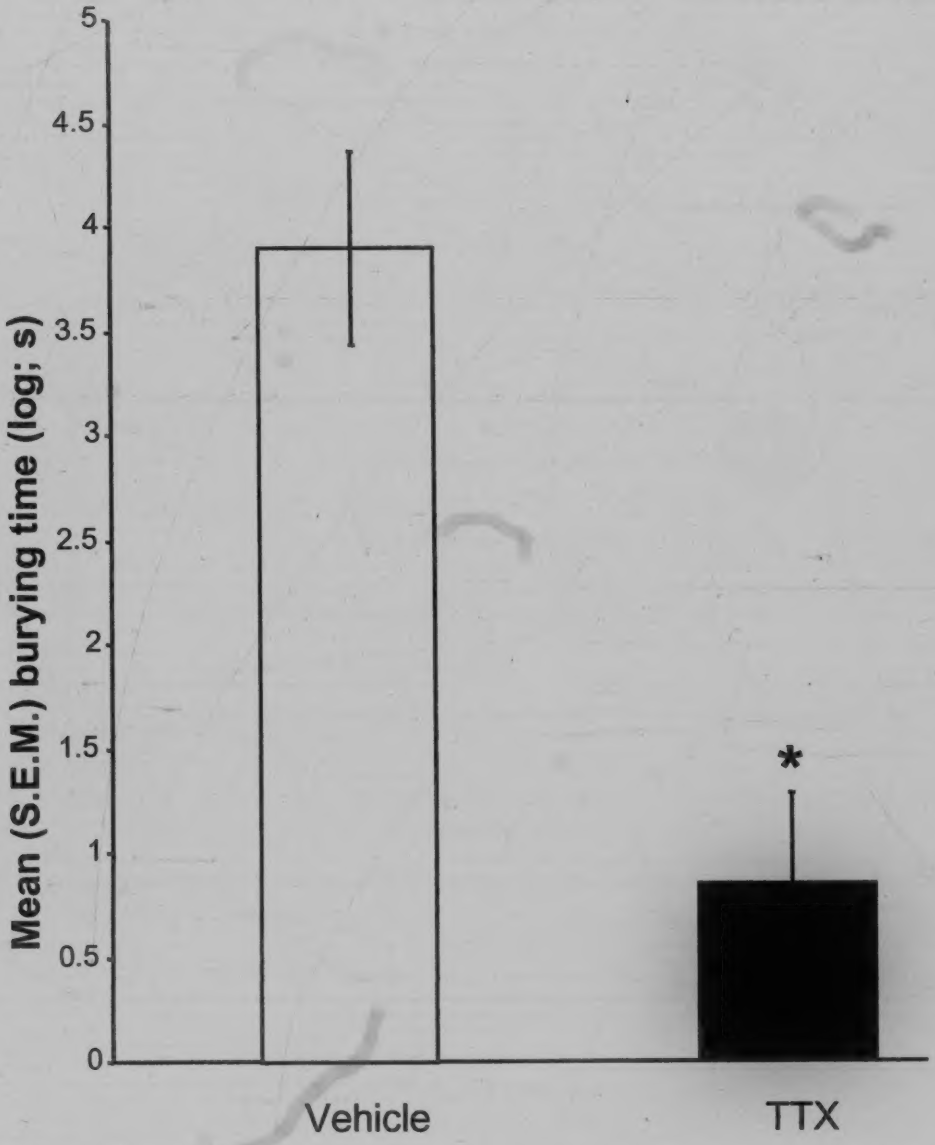

Mean (S.E.M.) burying time (log; s)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
TTX

Figure 2-7. Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites in Experiment 3. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from (Paxinos and Watson, 1986).

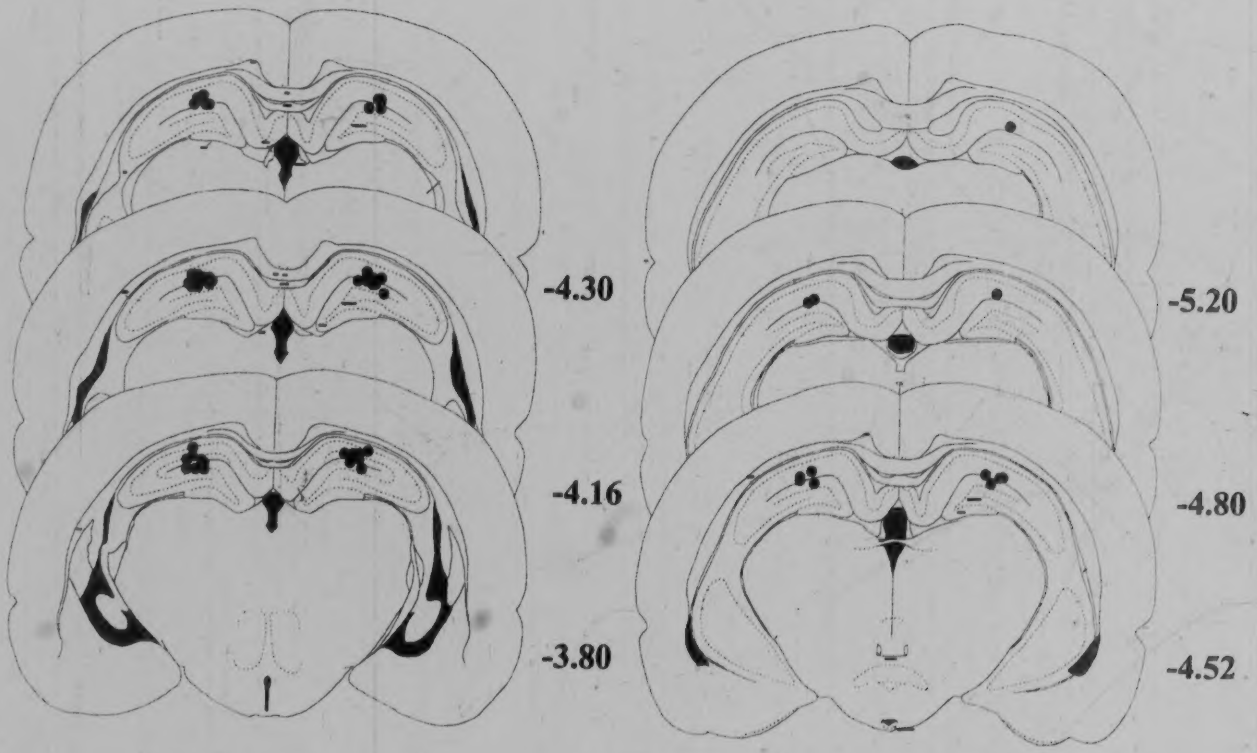
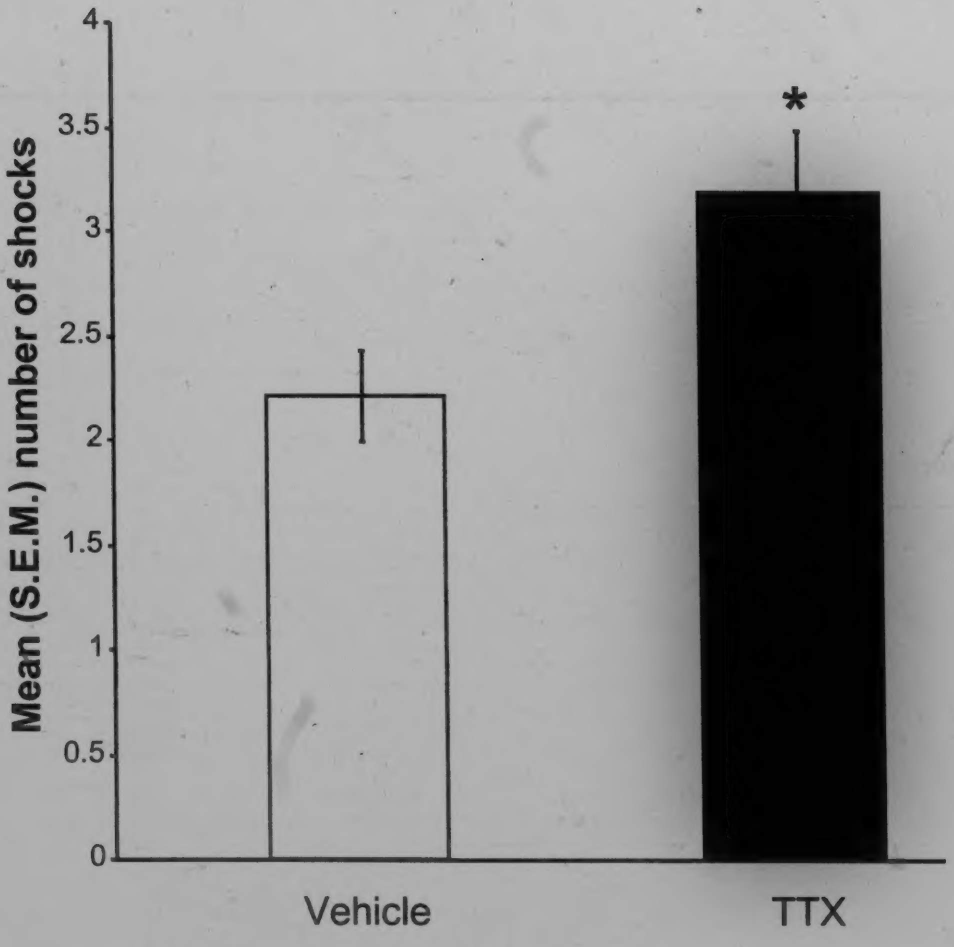

-4.30
-4.16
-3.80
-5.20
-4.80
-4.52

Figure 2-8. Mean (+/- S.E.M.) number of shocks in the shock probe apparatus after infusions of TTX (5 ng/0.5 µl) into the dorsal hippocampus. *p < .05 compared with the vehicle control group.



Mean (S.E.M.) number of shocks
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
TTX

Figure 2-9. Schematic illustration of coronal sections of the rat brain showing the approximate location of ventral hippocampal infusion sites in Experiment 4. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from (Paxinos and Watson, 1986).

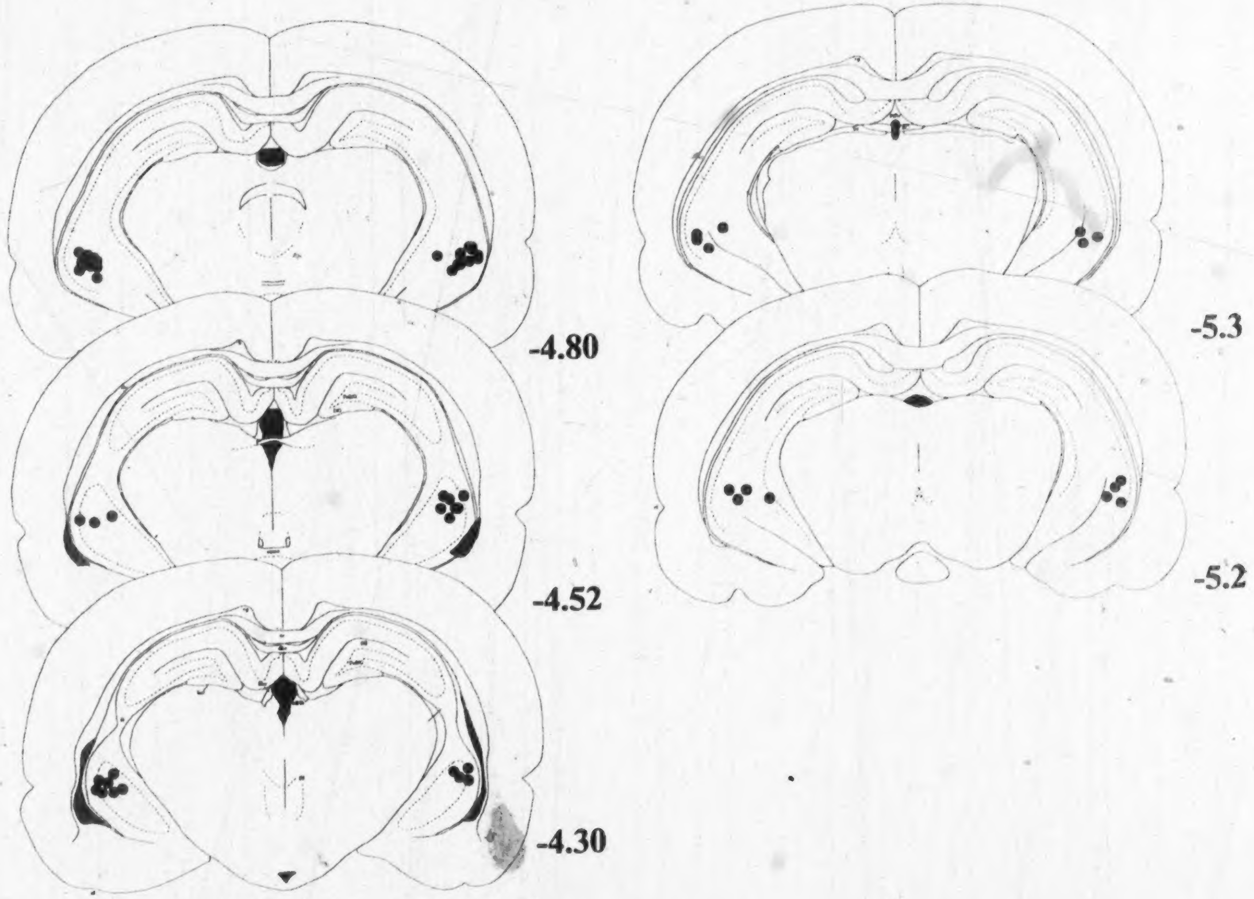

-4.80
-4.52
-4.30
-5.3
-5.2

Figure 2-10. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of TTX (5 ng/0.5 µl) into the ventral hippocampus. *p < .05 compared with the vehicle control group.

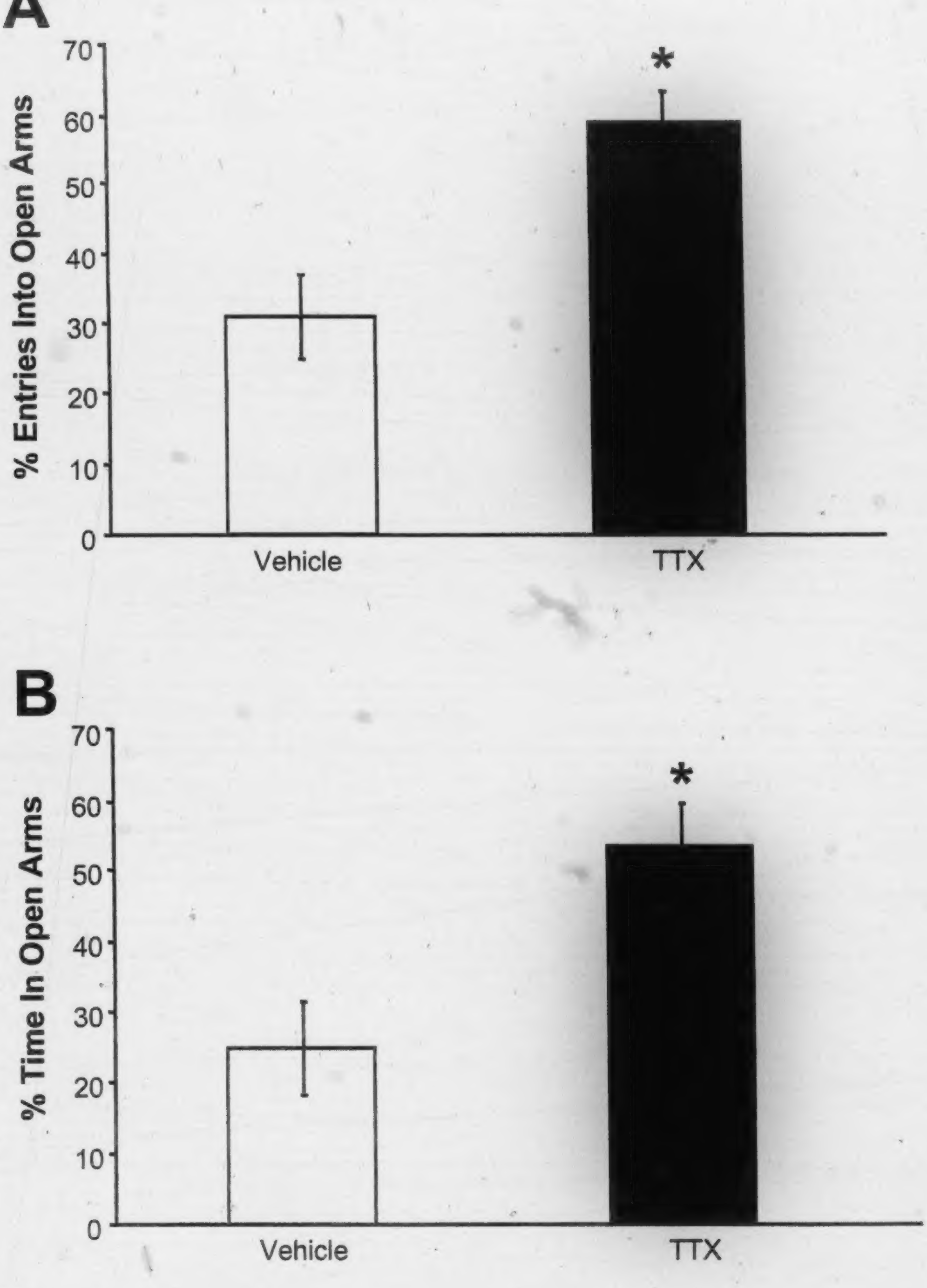
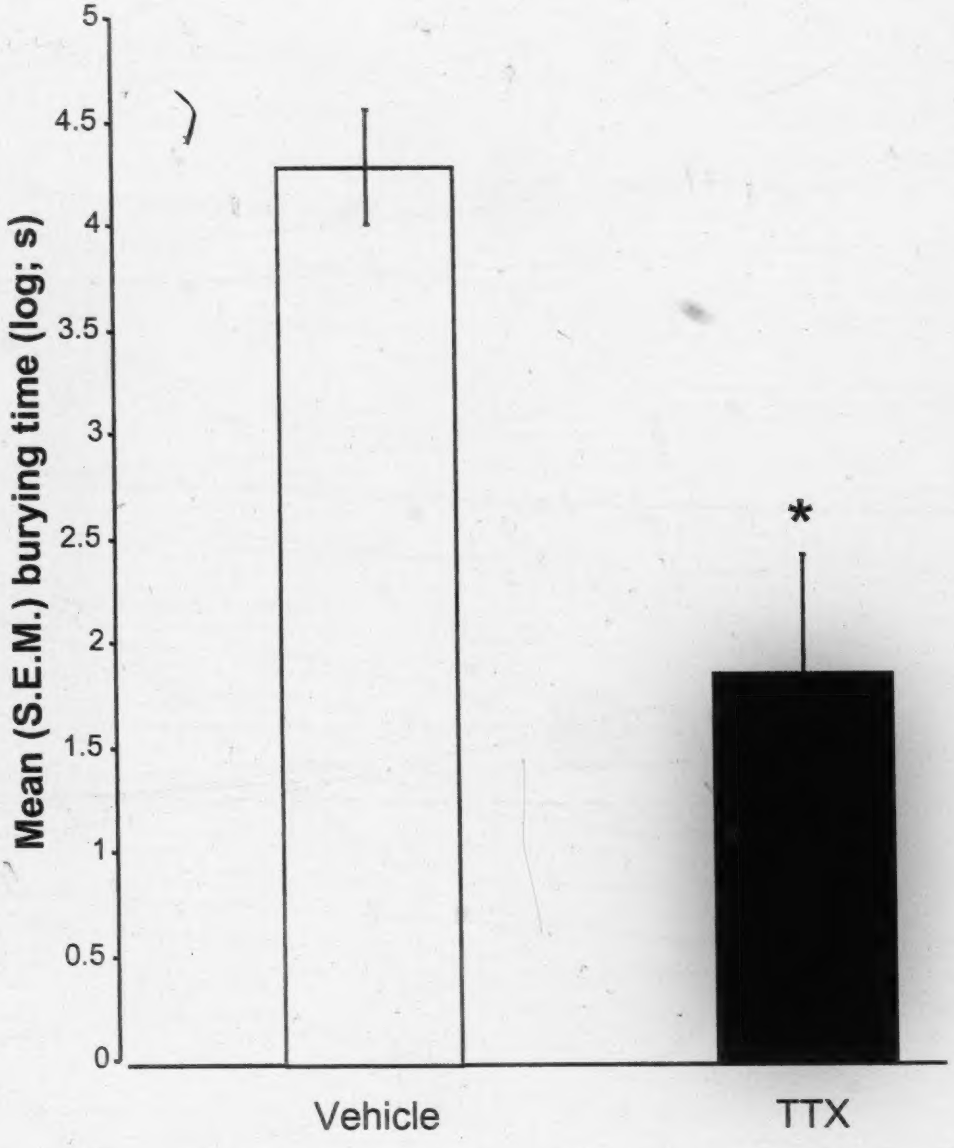

A
% Entries Into Open Arms
70
60
50
40
30
20
10
0
*
Vehicle
TTX
B
% Time In Open Arms
70
60
50
40
30
20
10
0
*
Vehicle
TTX

Figure 2-11. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of TTX (5 ng/0.5 µl) into the ventral hippocampus. *p < .05 compared with the vehicle control group.



Mean (S.E.M.) burying time (log; s)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
*
Vehicle
TTX

Figure 2-12. Labeled cell bodies and fibers following BDA injections in the dorsal hippocampus (A). There were no terminal regions in the amygdala following Injections of BDA in the dorsal hippocampus (B).

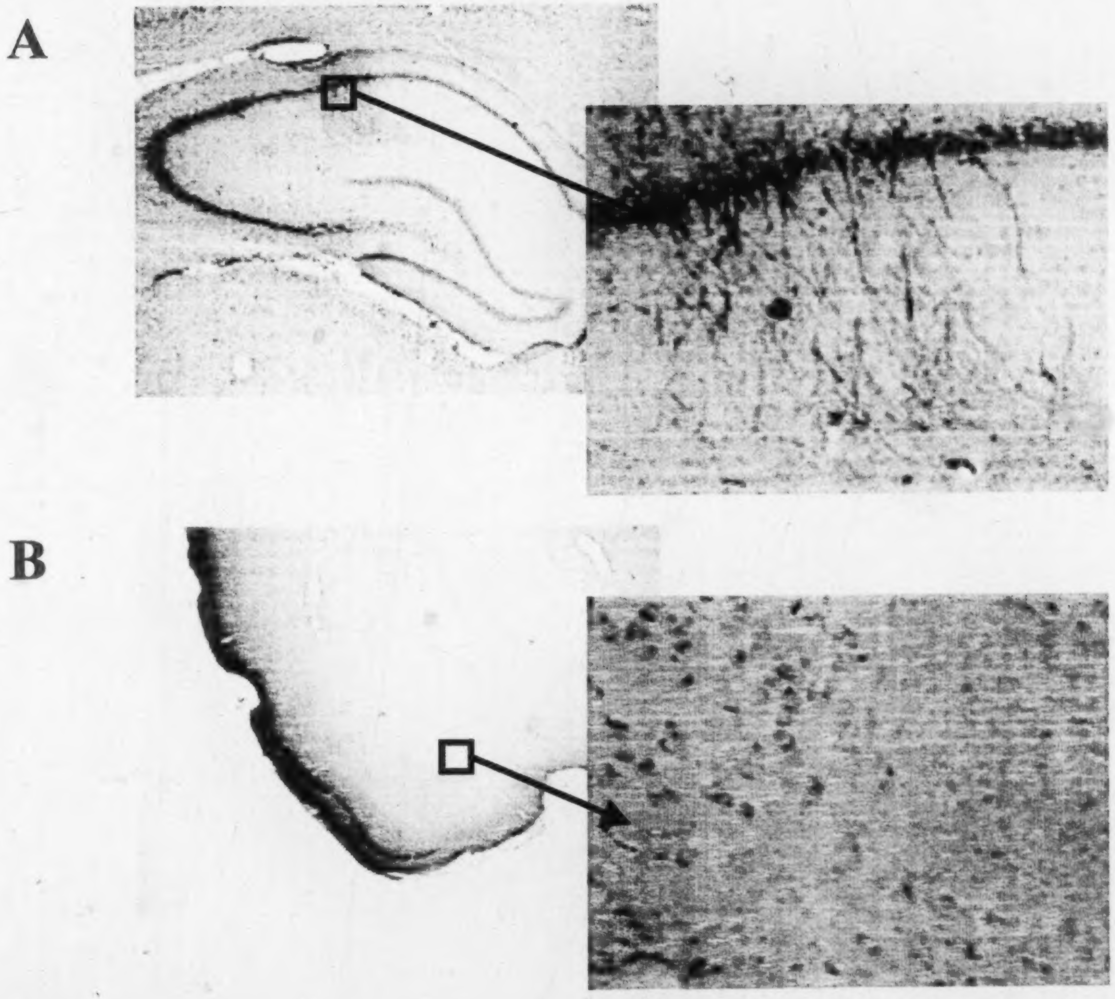
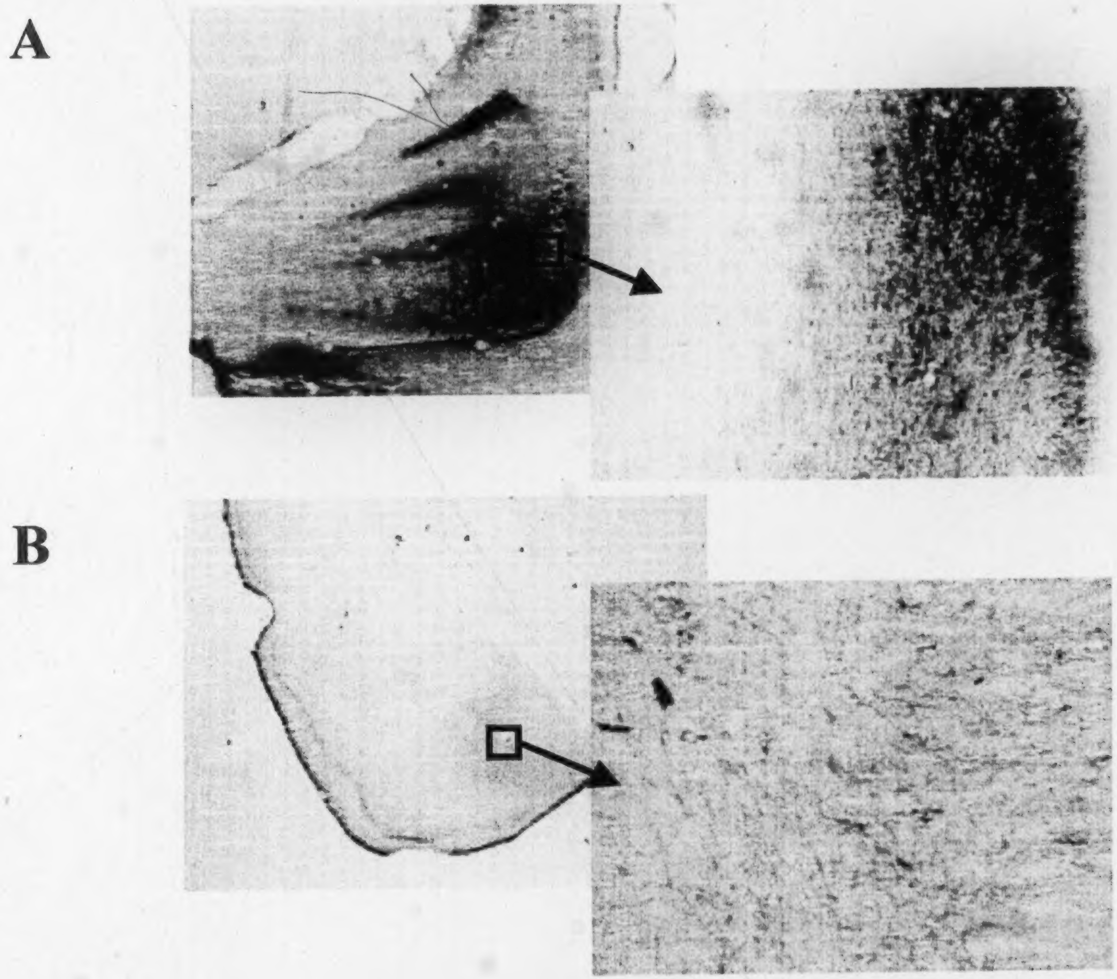

A
B

Figure 2-13. Labeled cell bodies and fibers following BDA injections in the ventral hippocampus (A). Densely labeled fibers in the amygdala were observed following Injections of BDA in the ventral hippocampus (B).


A
B

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Chapter 3

Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests.

A version of this chapter has been published in *Brain Research* (*Brain Research* 949: 60-70).

1. Introduction

Emerging evidence suggests that increased acetylcholine (ACh) levels results in decreased anxiety levels. Clinical evidence for the effect of acetylcholine on anxiety in humans comes mainly from patients with Alzheimer's disease. Alzheimer's is associated with decreased ACh levels and roughly 33% of Alzheimer's patients suffer from anxiety disorders (Weiner et al., 1997). More importantly, treatment with acetylcholinesterase (AChE) inhibitors decreased anxiety in Alzheimer's patients (Weiner et al., 1997). In addition, there is some evidence that nicotine reduces stress-induced anxiety in humans (Jarvik et al., 1989; Pomerleau et al., 1984).

Experimental evidence for the role of ACh in anxiety comes from animal models of anxiety. For example, systemic injections of the AChE inhibitor physostigmine decreased novelty-induced neophobia in rats (Sienkiewicz-Jarosz et al., 2000). Furthermore, both muscarinic and nicotinic receptors seem to be involved. Systemic injections of muscarinic antagonists increased anxiety in the plus-maze and black-white box tests (Rodgers and Cole, 1995; Smythe et al., 1996), whereas systemic injections of nicotinic agonists in rats or mice decreased anxiety in the elevated plus maze, social interaction, and contextual fear conditioning tests (Brioni et al., 1993; Decker et al., 1994; Irvine et al., 1999; Szyndler et al., 2001). It should be noted, however, that the effects of nicotine on experimental anxiety in rodents appear to depend on dose, injection test-interval and gender (Faraday et al., 1999; File et al., 1998; Irvine et al., 1999).

One brain structure that appears to modulate the effects of ACh on anxiety is the hippocampus. For example, increases in rats' fear reactions were observed in a variety of tests following intra-hippocampal infusions of both muscarinic and nicotinic antagonists (File et al., 1998; Hess and Blozovski, 1987; Smythe et al., 1998). Also, hippocampal cholinergic blockade enhances the hypothalamic-pituitary-adrenal response to stress (Bhatnagar et al., 1997). Lastly, we recently showed in our laboratory that infusions of physostigmine in the dorsal hippocampus decreased anxiety as measured in plus-maze and shock-probe tests (Degroot et al., 2001). Thus far, however, the anatomical specificity of this manipulation has not been explored.

The dorsal and ventral hippocampus have different afferent and efferent connections. Unlike the dorsal hippocampus, the ventral hippocampus receives extensive projections from, and sends projections to, various sub-nuclei of the amygdala (Pikkarainen et al., 1999; Van Groen and Wyss, 1990). In addition, the median raphe nucleus sends dense projections to the dorsal hippocampus while projecting more sparsely to the ventral hippocampus. Conversely, the dorsal raphé nucleus sends denser projections to the ventral hippocampus (Azmitia and Segal, 1978; McKenna and Vertes, 2001; Vertes et al., 1999). Lastly, commissural fibers between the CA1 regions exist in the dorsal, but not the ventral, hippocampus (Van Groen and Wyss, 1990). Therefore it would not be surprising if the dorsal and ventral hippocampus modulate anxiety differently. In fact, a distinction was observed following the infusion of serotonergic compounds in the hippocampal region. Infusions of the serotonin (5-HT) 1_A receptor agonist 8-hydroxy-2-(n-dipropylamino)tetraline hydrobromide (8-OH DPAT) in the ventral hippocampus had no effect on anxiety measures in the plus-maze test or social interaction (File and Gonzalez, 1996; Hogg et al., 1994). Conversely, the same dose of 8-OH DPAT, when infused in the dorsal hippocampus, induced a significant anxiogenic effect as measured in the plus-maze and social interaction tests (File et al., 1996).

To date, no studies have examined the role of the ventral hippocampal cholinergic system in fear and anxiety. In addition, the role of the dorsal hippocampal cholinergic system is still poorly understood (Degroot et al., 2001). The present study sought to further examine the role of the hippocampal cholinergic system in fear and anxiety. In particular, the relative importance of both the dorsal and ventral hippocampal cholinergic systems was compared. Cholinergic activity was increased using the AChE inhibitor physostigmine because of results obtained with this compound in previous studies (Degroot et al., 2001; Sienkiewicz-Jarosz et al., 2000) and because of the possible relevance of AChE inhibitors for the treatment of human anxiety (Cummings and Kaufer, 1996; Levy et al., 1999).

Anxiety was assessed in the elevated plus-maze and the shock-probe burying tests. In the elevated plus-maze test, rats typically avoid the open arms of the maze and spend most of their time in the two enclosed arms (Pellow et al., 1985). In the shock-probe burying test, rats shocked from a stationary, electrified probe push bedding material from the floor of the experimental chamber toward the shock-probe (i.e., burying) while avoiding further contacts with the probe (Pinel and Treit, 1978; Treit et al., 1981; Treit et al., 1994). Anxiolytic drugs such as diazepam increase open-arm exploration in the plus-maze and decrease burying toward the shock-probe (De Boer et al., 1990; Pellow, 1986; Pellow et al., 1985; Pellow and File, 1986; Treit and Pinel, 1981; Treit et al., 1994; Treit and Menard, 1997; Tsuda et al., 1988). Conversely, anxiogenic drugs such as yohimbine decrease open-arm exploration while increasing shock-probe burying (Johnston and File., 1989; Pellow, 1986; Pellow et al., 1985; Pellow and File, 1986; Treit., 1990; Tsuda et al., 1988). It is important to note that anxiolytic effects in shock-probe and plus-maze tests involve a decrease and an increase in activity, respectively. This suggests that reductions in anxiety in *both* tests would be difficult to explain in terms of non-specific effects on general activity, arousal, pain sensitivity, or behavioral inhibition. Furthermore, neither test involves response learning or an explicit memory requirement, factors that can complicate the interpretation of drug effects in other animal models of anxiety (e.g., the conflict test; Tsuda et al., 1988).

2. Materials and Methods

2.1. *Experiment 1*

2.1.1. *Subjects*

The subjects were naive, male albino Sprague-Dawley rats, purchased from Charles River, Canada, weighing 300-350 g at the time of surgery. Following surgery, rats were individually housed in polycarbonate cages (47 x 25 x 20.5 cm) and maintained on a 12:12 h light/dark cycle (lights on at 07.00h), with food and water available *ad libitum*. Behavioral testing occurred between 09.00 and 19.00 h. The treatment of all animals was in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.1.2. Surgery and Histology

The rats were given an oral administration of the analgesic acetaminophen (Tylenol 120 mg/1.5 ml) followed one hour later by atropine sulfate (0.1 mg/0.2 ml, i.p.) to reduce respiratory complications due to the anaesthetic. The rats were then anaesthetized with pentobarbital (Nembutal 50 mg/kg, i.p.), hydrated with saline (3 cc, s.c.), and given the antibiotic penicillin (Crystiben, Rhone Merieux Canada Inc., 15,000 I.U./0.05 cc, i.m.). Stereotaxic procedures were used to implant 40 rats with two 22 gauge stainless-steel guide cannulae (Plastics One, Inc. Roanoke, VA) aimed bilaterally to terminate 1 mm above the dorsal hippocampus (-4.2 mm AP, 2.0 mm D.V, +/- 4.1 mm lateral to the midline), using flat skull coordinates (Paxinos and Watson, 1986). The cannulae were attached to the skull with four jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula to keep the cannula tract clear. Immediately after surgery the rats were placed into a warm environment until they regained consciousness. Two days after surgery, each cannula was checked for obstructions, and betadine was applied to the surgical wound. Following behavioral testing, rats were sacrificed with an overdose of chloral hydrate and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and placed in a 10% formalin solution. After at least 48 hours had elapsed, the brains were frozen with dry ice, sectioned (60 μm), mounted onto glass slides and stained with thionin. The location of the cannulae for each rat was examined microscopically by an observer who was "blind" to the behavioral results. The location of the cannulae tips were then transcribed onto the appropriate atlas plates (Paxinos and Watson, 1986). The behavioral data for animals with misplaced cannulae (n = 2) were discarded. "Misplacements" occurred when either cannula was outside of the intended target. In addition, two rats failed to contact the probe in the shock-probe burying test and therefore were not included in the analyses of the burying data.

2.1.3. *Infusion Procedures*

Rats in each of the two surgical groups were randomly assigned to one of the following two drug conditions: (1) a control condition, infused with vehicle or (2) a drug condition, infused with physostigmine. The tame, hand-held rats were given an infusion of physostigmine (10 μg/0.5 μl/1 min) or its vehicle (phosphate buffered saline; PBS, pH 7.4; 0.5 μl/1 min) into the dorsal hippocampus through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula. The internal cannula was connected to a 10 μl constant rate Hamilton microsyringe with polyethylene tubing and the infusions were delivered using an infusion pump (Harvard Apparatus 22). The internal cannula was left in place for one minute following the infusions in order to allow for diffusion. The physostigmine dose was selected based on previous work (Degroot et al., 2001) as well as pilot work in our laboratory.

2.1.4. *Behavioral Testing*

The behavioral testing procedures were the same as those used in previous experiments (Meanrd and Treit, 2000; Pesold and Treit, 1996; Treit et al., 1993; Treit et al., 1994). All behavior was recorded on videotape for ensuing analysis. Plus-maze testing occurred at least seven days post-surgery and shock-probe testing occurred at least 12 days post-surgery. This order of testing is based on earlier work (Treit and

Pesold, 1990) showing no effect of a 5 minute exposure to the plus-maze on subsequent behavior in the shock-probe test, but some disruption of plus-maze behavior when preceded by a shock-probe test. Drug conditions (vehicle versus physostigmine) were counterbalanced across the two behavioral tests.

2.1.5. *Plus-maze*

This wooden, plus-shaped apparatus was elevated to a height of 50 cm, and consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, each with an open roof. The maze was in the center of a quiet and dimly lit room. The rats' behavior was observed using a mirror that was suspended at an angle above the maze. Behavioral data were collected by a "blind" observer who sat quietly one meter behind one of the closed arms of the maze. Five minutes following their respective drug treatment, rats were placed individually in the center of the plus-maze, facing one of the closed arms. During the five-minute test period, the observer measured (1) time spent in the open arms, (2) time spent in the closed arms, (3) number of entries into the open arms, and (4) number of entries into the closed arms. An entry was defined as all four paws in the arm. The maze was cleaned with distilled water after each rat was tested. For the purpose of analysis (Pellow et al., 1985; Pellow and File, 1986), open-arm activity was quantified as the amount of time that the rat spent in the open arms relative to the total amount of time spent in any arm (open/total x 100), and the number of entries into the open arms was quantified relative to the total number of entries into any arm (open/total x 100). The total number of arms entered as well as the total number of closed arms entered were used as indices of general activity (for details see Pellow et al., 1985; Rodgers and Johnson, 1995).

2.1.6. *Shock-probe*

The shock-probe burying apparatus consisted of a 40 x 30 x 40 cm plexiglass chamber, evenly covered with approximately 5 cm of bedding material (odor-absorbent kitty litter). The shock-probe was inserted through a small hole on one wall of the chamber, 2 cm above the bedding material. The plexiglass shock-probe (6.5 cm long and 0.5 cm in diameter) was helically wrapped with two copper wires through which an electric current was administered. The AC current from the shock source was varied with potentiometers and set at 2 mA. Rats were habituated in pairs to the test chamber without the shock-probe, for 30 minutes on each of four consecutive days prior to the test day. On the test day, five minutes following their respective drug treatments, rats were individually placed in one corner of the testing chamber, facing away from the shock-probe. The probe was not electrified until the rat touched it with its snout or forepaws, at which point the rat received a brief, 2 mA shock. The 15-minute testing period began once the rat received its first shock and the probe remained electrified for the remainder of the testing period. Following the first shock, the duration of time each rat spent spraying bedding material toward or over the probe with its snout or forepaws (i.e. burying behavior) was measured, as was the total number of contact-induced shocks each rat received from the probe. An index of the rat's reactivity to each shock was scored according to the following four-point scale (Pesold and Treit, 1992): (1) flinch involving only head or forepaw, (2) whole body flinch, with or without slow ambulation away from the probe, (3) whole body flinch, and/or jumping, followed by immediate ambulation away from the probe, and (4) whole body flinch and jump (all four paws in the air), followed by immediate and rapid ambulation (i.e. running) to the opposite end of the chamber. A mean shock reactivity score was calculated for each rat by summing its shock reactivity scores and dividing by the total number of shocks it received. Finally, the total time that the rat spent immobile (e.g. resting on the chamber floor) during the 15-minute testing period was determined. All behavioral measures were made by a "blind" observer who was watching the rat via a video monitor in a room adjacent to the testing room.

2.1.7. *Statistical Analysis*

Results are expressed as means and standard errors of the mean (S.E.M.). The plus-maze data and the shock-probe data were analyzed using analysis of variance (ANOVA). In order to correct for non-normality and heterogeneity of variance, the burying scores were transformed to their logarithms prior to ANOVA. An alpha level of 0.05 was used as the criterion for statistical significance.

2.2. *Experiment 2*

The purpose of experiment 2 was to determine if the cholinergic systems of the dorsal and ventral hippocampus modulate anxiety differently. A difference was expected based on anatomical and pharmacological distinctions between the two hippocampal regions (Azmitia and Segal., 1978; File et al., 1996; File and Gonzalez, 1996; Hogg et al., 1994; Pikkarainen et al., 1999). The procedures used in this experiment were the same as those used in Experiment 1 with the following exceptions.

2.2.1 *Surgery*

Stereotaxic procedures were used to implant 40 rats with two 22 gauge guide cannulae aimed bilaterally to terminate 1mm above the ventral hippocampus (-5.2

mm AP, 5.7 mm DV, +/- 5.6 mm lateral to the midline), using flat skull coordinates (33). The behavioral data for animals with misplaced cannulae (n = 5) was discarded. An additional seven rats in plus-maze and nine rats in shock-probe could not be tested due to difficulties with the infusion procedure.

2.2.2. *Procedure*

The tame, hand-held rats were given an infusion of physostigmine (10 μg/0.5 μl/1 min) or its vehicle (PBS; 0.5μl/1 min) into the ventral hippocampus through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula. The infusions were delivered using an infusion pump (Harvard Apparatus 22). The 10 μg dose of physostigmine was selected based on the findings from Experiment 1 and pilot work in our laboratory. Rats were randomly assigned to one of the two drug conditions: (1) vehicle in the hippocampus or (2) physostigmine in the hippocampus.

3. Results

3.1. *Experiment 1*

3.1.1. *Plus-maze*

Figure 3-1 shows the infusion sites in the dorsal hippocampus. Figure 3-2 indicates that physostigmine infusions into the dorsal hippocampus produced clear anxiolytic-like effects in the plus-maze. Specifically, rats infused with 10 μg of physostigmine into the dorsal hippocampus showed a significantly greater percentage of open-arm entries [$F(1,36) = 11.69$, $p = 0.002$] and open-arm time [$F(1,36) = 5.43$, $p = 0.026$] than their vehicle-infused controls. There was no indication of non-specific changes in general activity, since neither the total number of arms entered [$F(1,36) =$

0.001, p = 0.98; see table 3-1] nor closed arm entries [F(1,36) = 3.44, p = 0.072; see table 3-1] differed between groups.

3.1.2. *Shock-probe*

Figure 3-3 indicates that physostigmine infusions also produced anxiolytic-like effects in the shock probe burying test. Rats infused with 10 μg of physostigmine in the dorsal hippocampus displayed significantly [F(1,32) = 25.28, p = 0.000] lower burying levels than their respective vehicle-infused controls. This anxiolytic-like effect occurred in the absence of any significant changes in immobility [F(1,32) = 2.02, p = 0.17], number of shocks received [F(1,32) = 1.85, p = 0.18], or shock reactivity [F(1,32) = 0.88, p = 0.36; see table 1]. Taken together, these data suggest that increasing extracellular ACh levels with a 10 μg dose of physostigmine, in the dorsal hippocampus, produces behaviorally specific anxiolytic-like effects in both the plus-maze and shock-probe burying tests.

3.2. Experiment 2

3.2.1. *Plus-maze*

Figure 4 shows the location of the infusion sites in the ventral hippocampus. Infusions of physostigmine in the ventral hippocampus increased the percentage of open arm entries [F(1,26) = 4.39, p = 0.046] (figure 3-5, panel A) and open arm time [F(1,26) = 5.057, p = 0.033] (figure 3-5, panel B) compared to the vehicle-infused control group. Although physostigmine produced a significant reduction in closed arm entries compared to vehicle infused controls [F(1,26) = 4.56, p = 0.042; see table 3-1], it is difficult to attribute this difference to a reduction in general activity. First, the total number of arms entered did not differ significantly between the groups

[F(1,26) = 2.12, p = 0.16; see table 3-1], and second, physostigmine-infused animals entered significantly more open arms than vehicle-infused controls [F(1,26) = 4.39, p = 0.046]. If infusions of physostigmine had reduced general activity levels, then the total arm entries and open arms entered should also have been reduced. In our experiment, rats infused with physostigmine were more likely to enter the open arms and less likely to enter the closed arms than vehicle infused controls. Thus the effect on closed arm entries was likely due to anxiety reduction, rather than to reduced general activity.

3.2.2. *Shock-probe*

Figure 6 shows that infusions of physostigmine in the ventral hippocampus also reduced burying behavior. Physostigmine-infused rats buried the shock-probe significantly less than vehicle controls [F(1,24) = 7.75, p = 0.010]. Interestingly, unlike infusions of physostigmine in the dorsal hippocampus, infusions in the ventral hippocampus also affected the number of shocks received. More specifically, rats infused with physostigmine in the ventral hippocampus took significantly more shocks than vehicle infused controls [F(1,24) = 20.15, p = 0.001; see figure 3-7]. These anxiolytic effects were not confounded by between-group differences in general activity [F(1,24) = 1.38, p = 0.25; see table 3-1], although there was a significant between-group difference in shock reactivity [F(1,24) = 5.32, p = 0.030; see table 3-1]. In order to determine if shock reactivity contributed to the significant differences in burying activity or shock number between the groups, we performed analyses of covariance using shock reactivity as the covariate. These analyses showed that shock reactivity did not contribute significantly to the number of shocks taken by the rats [F(1,23) = 10.41, p = 0.004], or to the duration of burying [F(1,23) = 11.17, p = 0.003].

4. General Discussion

The results of the present study indicate that increasing cholinergic levels in the dorsal or the ventral hippocampus significantly reduces anxiety as measured in the plus-maze and shock-probe tests. The anxiolytic-like effects seen in our study are difficult to explain in terms of non-specific drug effects on general activity, arousal, or behavioral inhibition since anxiety-reduction in the plus-maze is mainly indicated by a selective increase in a specific activity (open arm exploration), while anxiety-reduction in shock-probe is mainly indicated by a selective decrease in a specific activity (burying behavior). Furthermore, both tests utilize rats' untrained fear reactions to clear and present anxiogenic stimuli, making interpretations in terms of learning or memory processes unlikely.

Interestingly, there were some differences in behavior between rats infused in the dorsal hippocampus compared to rats infused in the ventral hippocampus. Rats infused with physostigmine in the ventral (but not the dorsal) hippocampus took significantly more shocks. This suggests that cholinergic receptors in the ventral, but not the dorsal hippocampus, are involved in passive avoidance of painful stimuli.

The anxiolytic effects induced by stimulating the hippocampal cholinergic system are consistent with previous data, which indicate that intra-hippocampal infusions of cholinergic antagonists increase anxiety (File et al., 1998; Hess and Blozovski, 1987; Smythe et al., 1998). Our data also reinforce previous work in our laboratory, which showed that unilateral infusions of physostigmine in the dorsal hippocampus significantly reduce anxiety measures (Degroot et al., 2001). Unlike intra-hippocampal infusions of nicotine, which has been found to be anxiolytic, anxiogenic, or without effect in animal models of anxiety (File et al., 1998; Ouagazzal et al., 1999), intra-hippocampal infusions of physostigmine in our study produced consistent results. The inconsistencies obtained with intra-hippocampal infusions of nicotine may be due to differences between animal testing procedures, differences in basal "anxiety" levels, or to differences in the relative specificity of cholinergic manipulations. In our study it is likely that all cholinergic receptor sub-types would have been stimulated simultaneously, which resulted in a net anxiolytic effect.

It is very unlikely that rats infused with physostigmine in the ventral hippocampus failed to avoid the shock-probe because they were unable to associate the shock with the probe. Previous studies indicate that increasing cholinergic levels in the hippocampus enhance rather than impair learning and memory. For instance, increased ACh output in the hippocampus was positively correlated with enhanced spatial and reference memory (Fadda et al., 2000; Kopf et al., 2001; Stancampiano et al., 1999). Also, in mice, intra-hippocampal infusion of cholinergic agonists enhanced retention following footshock avoidance training in a T-maze (Farr et al., 2000). Conversely, decreased ACh concentrations, decreased ACh release, decreased choline acetyl transferase activity, or the administration of nicotinic antagonists in the hippocampus have been associated with learning and memory deficits in the T-maze, the Morris water maze, delayed non matching to position, passive avoidance, and the radial arm maze (Felix and Levin, 1997; Lehmann et al., 2000; McDonald et al., 1998; Tateishi et al., 1987).

While the roles of GABA and serotonin in fear and anxiety have been studied extensively over the past 30 years (e.g. Argyropoulos et al., 2000; Bourin and Hascoet, 2001), until recently the role of ACh in anxiety has received relatively little attention. Our study is important, in this regard, because it provides clear support for a role of ACh in anxiety modulation and also confirms that the hippocampus is an important site of action for the anxiolytic effect of ACh. In addition, our study is the first to directly compare the importance of cholinergic processes in the ventral and dorsal hippocampi in anxiety modulation. Finally, the dissociation we found in shock-probe avoidance indicates that there is some degree of anatomical specificity in the effects of cholinergic manipulations on different anxiety responses.

At the same time, the exact mechanism whereby hippocampal cholinergic stimulation modulates anxiety remains to be determined. It is possible that the hippocampus affects anxiety through an interaction with other brain structures, such as the septum. Previous work in our laboratory has repeatedly shown that pharmacological (i.e. GABAergic) inhibition of the septum reduces anxiety (Degroot et al., 2001; Pesold and Treit, 1992; Pesold and Treit, 1994; Pesold and Treit, 1996). The hippocampus sends a GABAergic projection to the medial septum and a glutamatergic projection to the lateral septum. The GABAergic projection originates from non-pyramidal cells in the stratum oriens of the CA1-CA3 region and innervates cholinergic and non-cholinergic neurons (Amaral and Witter, 1995). The glutamatergic pathway projects from pyramidal cells and terminates on GABAergic neurons of the lateral septum (Walass and Fonnum, 1980). Stimulating cholinergic receptors in the hippocampus could excite both the GABAergic and the glutamatergic projections. An activation of the GABAergic projection would likely result in a decrease of septal activity. Similarly, exciting the glutamatergic pathway would excite the GABAergic cells of the lateral septum and also result in a reduction of septal activity. In either case we would expect anxiety reduction based on previous results (Degroot et al., 2001; Drugan et al., 1986; Menard and Treit, 1996; Pesold and Treit, 1992; Pesold and Treit, 1994; Pesold and Treit, 1996).

In the present study, increasing ACh levels in the ventral, but not the dorsal, hippocampus affected the number of shocks received by the rats. It is possible that this difference is modulated through an interaction with the amygdala (Pikkarainen et al., 1999). Unlike the dorsal hippocampus, the ventral hippocampus is extensively connected with the amygdala (Pikkarainen et al., 1999; Van Groen and Wyss, 1990). Previous studies have shown that pharmacological or physiological inhibition of the amygdala significantly increases the number of shocks taken by rats placed in the shock probe apparatus (Pesold and Treit, 1994; Pesold and Treit, 1995; Treit et al., 1993). Thus, stimulating the ventral hippocampal cholinergic receptors could have increased shock-probe contacts by reducing activity in the amygdala.

In summary, the present findings demonstrate that infusions of the AChE inhibitor physostigmine into the dorsal or ventral hippocampus significantly reduced anxiety. These findings provide further support for the importance of ACh in anxiety modulation and the potential importance of AChE inhibitors in clinical studies. Both the dorsal and ventral hippocampus affect passive avoidance of non-painful stimuli (open arms), and active avoidance of painful stimuli (burying behavior). In addition, infusions of physostigmine in the ventral hippocampus also affect passive avoidance of painful stimuli (shock probe). The reason for this distinction remains unclear, but could result from a difference in anatomical connections.

Table 3-1. Mean (+/- S.E.M.) total arm entries and closed arm entries in the plus-maze task, and mean (+/- S.E.M..) activity and reactivity in the shock-probe burying test.

	Plus-maze			
	Dorsal Hippocampus		Ventral Hippocampus	
	PBS (n = 18)	PHYS (n = 20)	PBS (n = 14)	PHYS (n = 14)
Total arm entries	10.28	10.25	10.00	8.14
	(0.99)	(0.75)	(0.81)	(0.99)
Closed arm entries	8.78	7.00	7.29	5.07
	(0.69)	(0.66)	(0.76)	(0.71)
	Shock-probe			
	(n = 18)	(n = 16)	(n = 15)	(n = 11)
Immobility (s)	45.22	102.89	47.47	101.91
	(13.91)	(40.15)	(11.17)	(30.42)
Shock reactivity	2.29	2.16	2.09	1.69
	(0.07)	(0.50)	(0.13)	(0.10)
Shock number	1.83	2.25	1.80	6.10
	(0.19)	(0.25)	(0.22)	(1.28)

Figure 3-1. Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from (Paxinos and Watson, 1987).

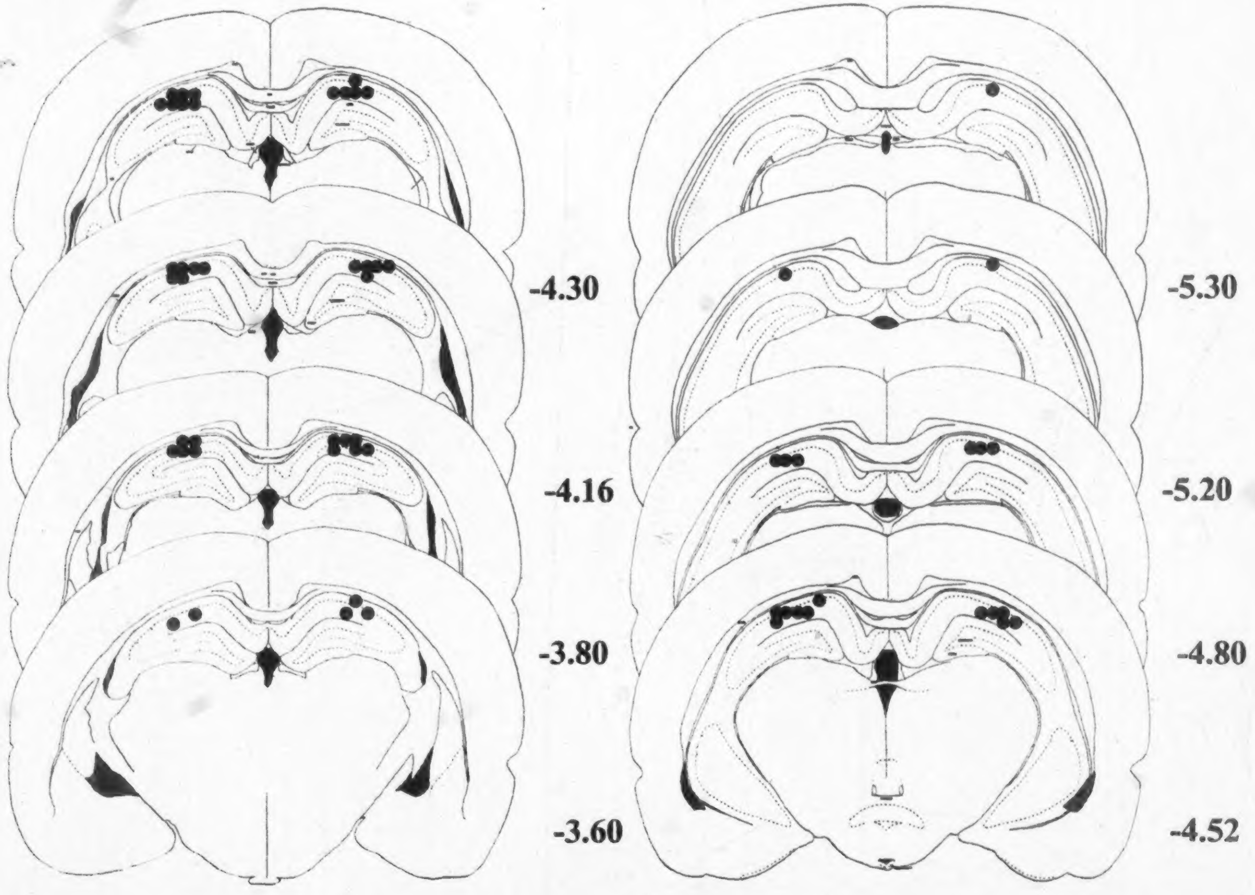

-4.30
-4.16
-3.80
-3.60
-5.30
-5.20
-4.80
-4.52

Figure 3-2. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of physostigmine (10 ug/0.5 μl) or vehicle (0.5 μl) into the dorsal hippocampus. *$p < .05$ compared with the vehicle control group.

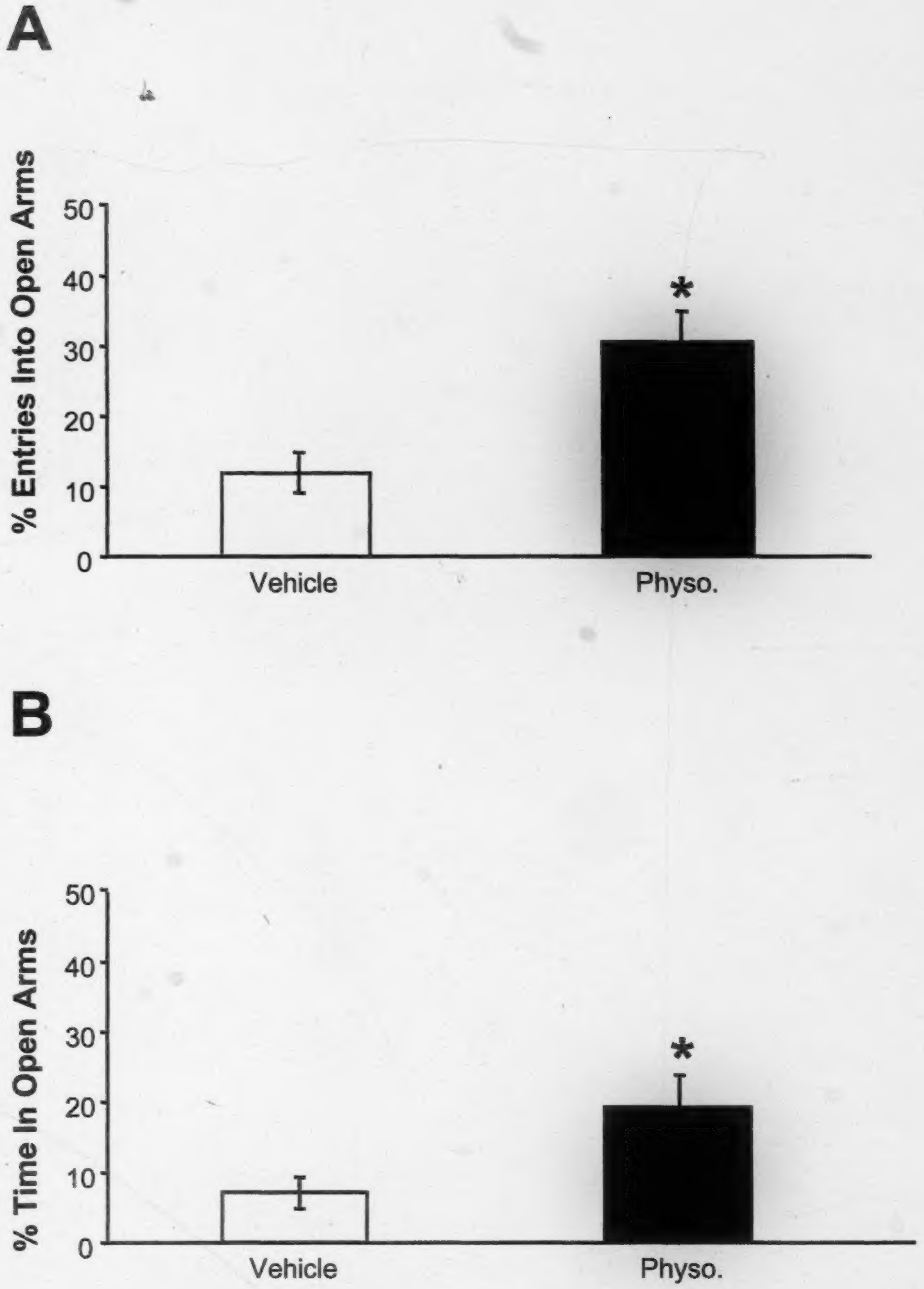
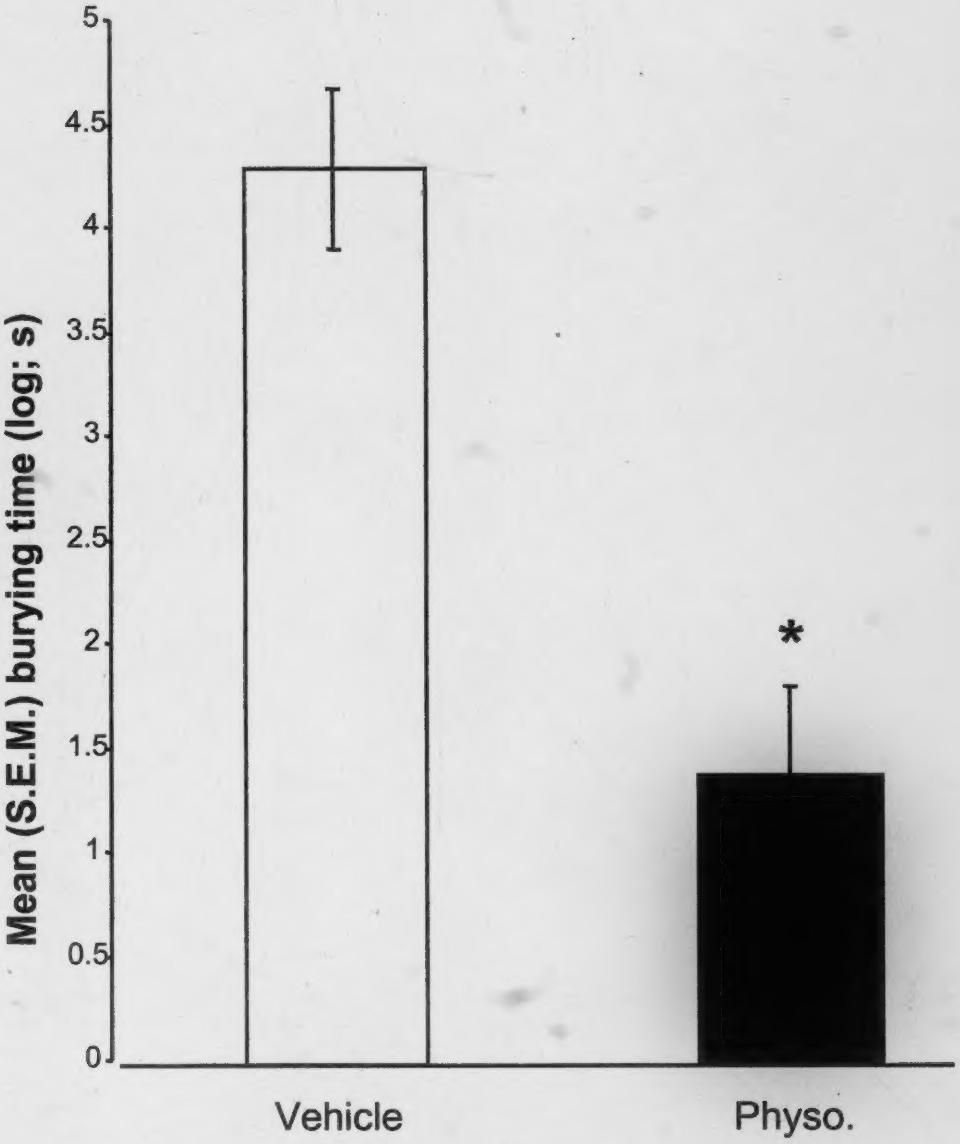

A
% Entries Into Open Arms
50
40
30
20
10
0
*
Vehicle
Physo.
B
% Time In Open Arms
50
40
30
20
10
0
*
Vehicle
Physo.

Figure 3-3. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (10 ug/0.5 µl) or vehicle (0.5 µl) into the dorsal hippocampus. *p < .05 compared with the vehicle control group.



Mean (S.E.M.) burying time (log; s)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
Physo.

Figure 3-4. Schematic illustration of coronal sections of the rat brain showing the approximate location of ventral hippocampal infusion sites in Experiment 2. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from (Paxinos and Watson, 1987).

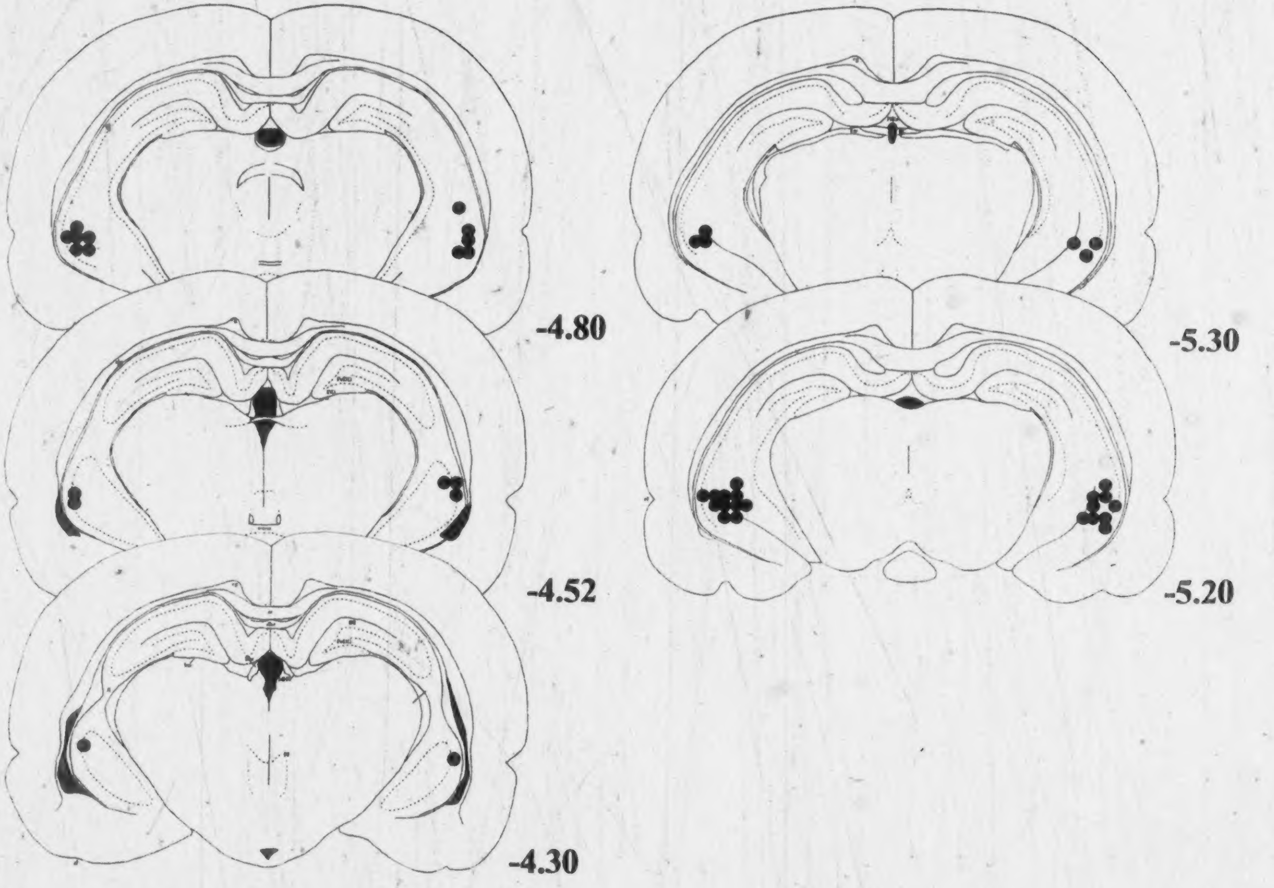

-4.80
-4.52
-4.30
-5.30
-5.20

Figure 3-5. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of physostigmine (10 ug/0.5 μl) into the ventral hippocampus. *p < .05 compared with the vehicle control group.

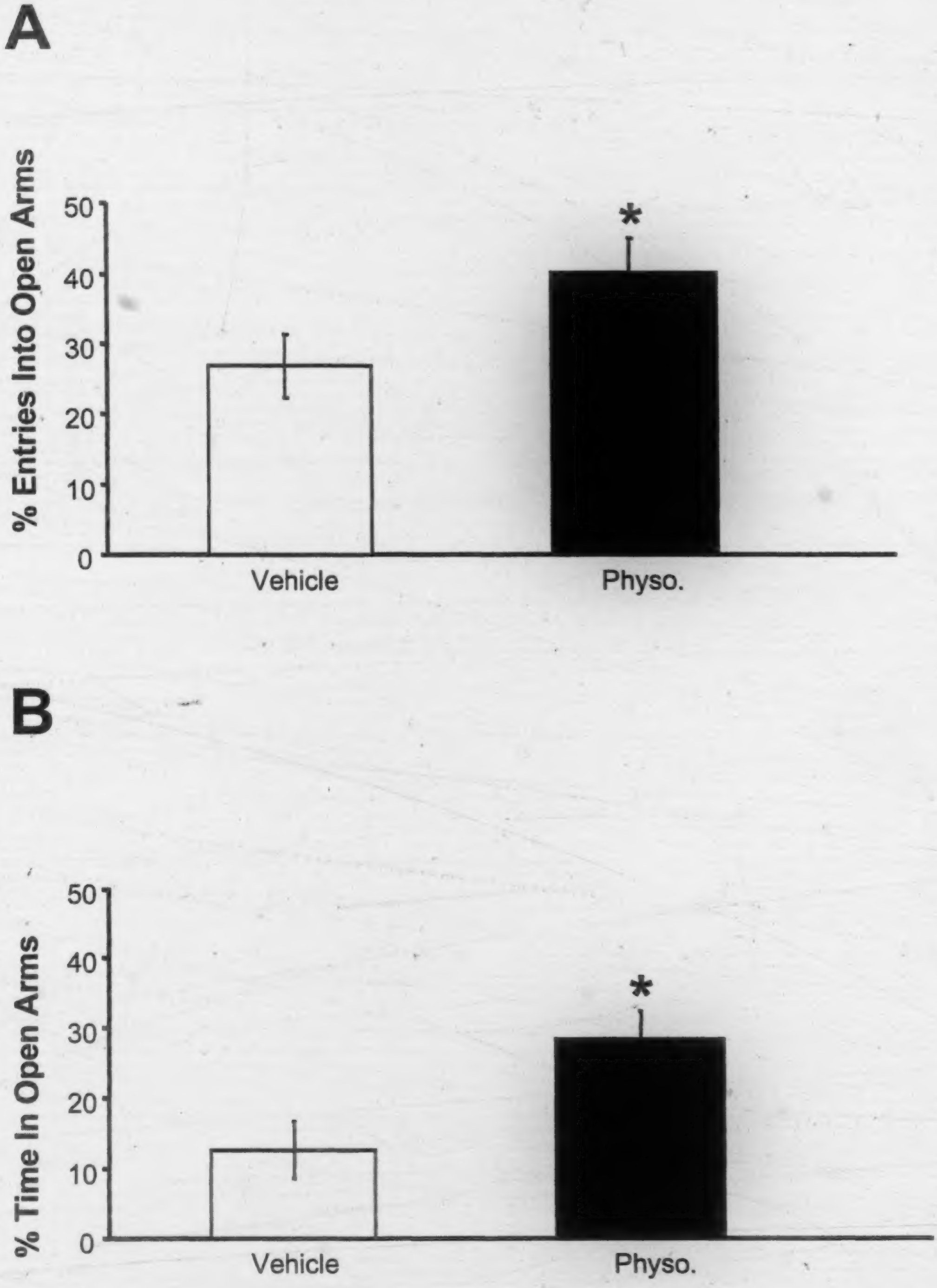

A
% Entries Into Open Arms
50
40
30
20
10
0
*
Vehicle
Physo.
B
% Time In Open Arms
50
40
30
20
10
0
*
Vehicle
Physo.

Figure 3-6. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (10 µg/0.5 µl) into the ventral hippocampus. *p < .05 compared with the vehicle control group.

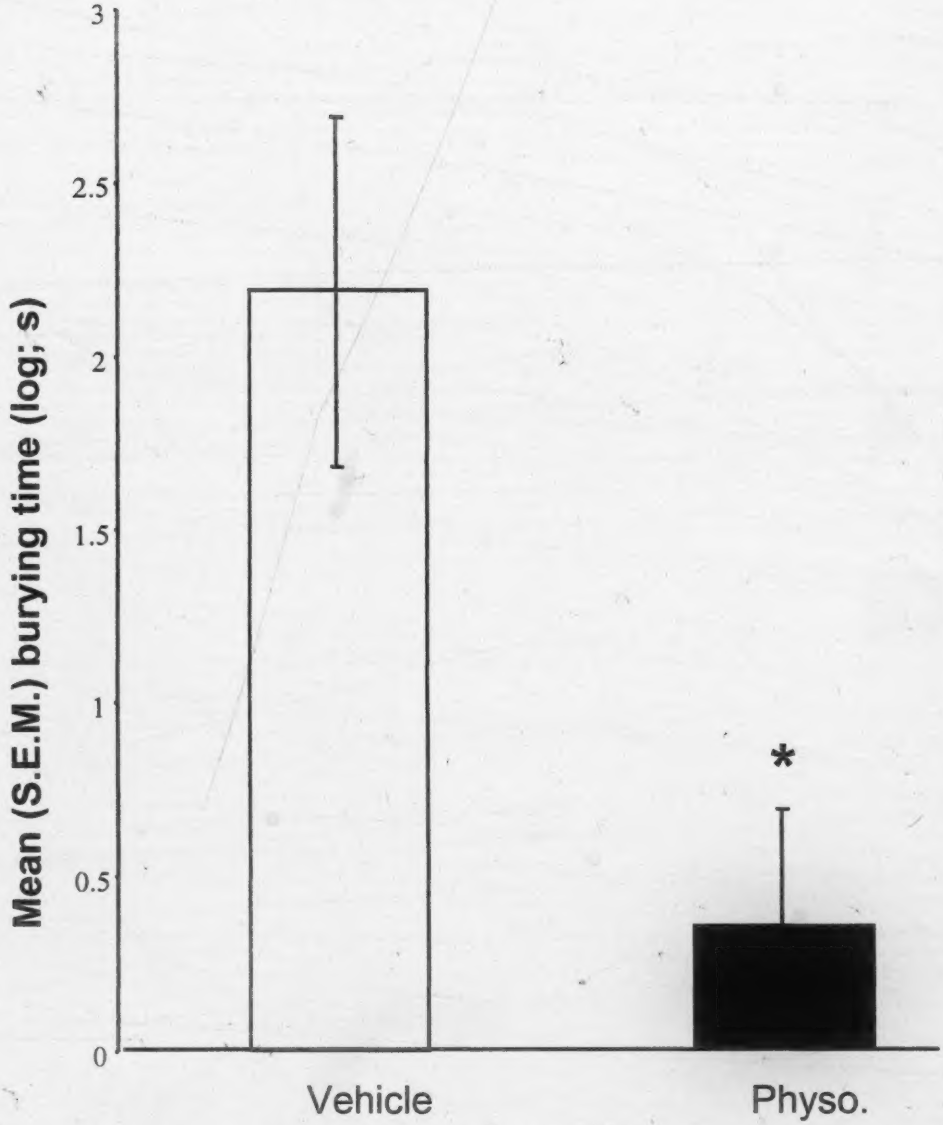
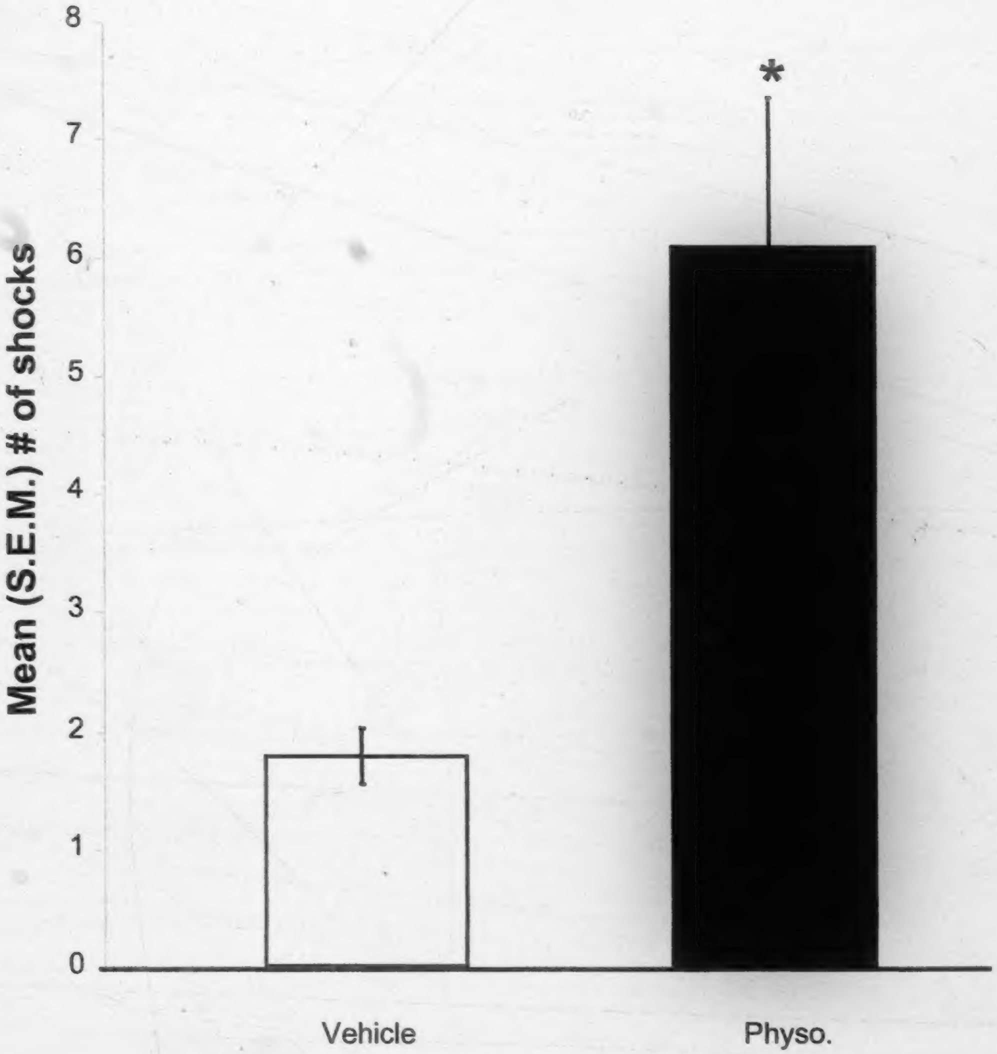

Mean (S.E.M.) burying time (log; s)
3
2.5
2
1.5
1
0.5
0
*
Vehicle
Physo.

Figure 3-7. Mean (+/- S.E.M.) number of shocks in the shock probe apparatus after infusions of physostigmine (10 µg/0.5 µl) into the ventral hippocampus. *p < .05 compared with the vehicle control group.


Mean (S.E.M.) # of shocks
0
1
2
3
4
5
6
7
8
*
Vehicle
Physo.

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Chapter 4

Septal GABAergic and Hippocampal Cholinergic Systems Modulate Anxiety in the Plus-maze and Shock-probe Tests.

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Introduction

Anatomically, the septum is extensively connected to the hippocampus (e.g., Nauta & Domesick, 1982; Risold & Swanson, 1997; Swanson & Cowan, 1977), and together they form a substantial part of the limbic system. Functionally, according to Gray's theory (Gray, 1982; 1991), the septum and hippocampus act in concert to control anxiety, as evidenced in part by the remarkable correspondence between the effects of septal or hippocampal lesions in traditional aversive learning paradigms and the effects of anxiolytic drugs in the same paradigms (for reviews, see Gray, 1982; Gray & McNaughton, 1983).

With respect to the septum, we have repeatedly found that ablation or pharmacological inhibition of this area reduces rats' anxiety-related behaviors in the elevated plus-maze and the shock-probe burying tests, suggesting that the septum normally plays an excitatory role in the control of anxiety (for reviews see Treit & Menard, 2000; Menard & Treit, 1999). Briefly, we found that electrolytic or excitotoxic lesions of the septum produced anxiolytic-like effects in the plus-maze and shock-probe burying tests, i.e., open-arm exploration was increased and burying behavior was decreased (Menard & Treit, 1996a,b; Pesold & Treit, 1992; Treit & Pesold, 1990; Treit, Pesold, & Rotzinger, 1993a). The same pattern of effects was produced when septal activity was inhibited via intra-septal infusions of the benzodiazepine-type anxiolytic, midazolam (Pesold & Treit, 1994, 1996). Although benzodiazepine anxiolytics are thought to produce their effects indirectly through an allosteric modulation of the $GABA_A$ receptor complex (e.g., Zorumski & Isenberg, 1991), there is surprisingly little evidence to suggest that directly acting $GABA_A$ agonists such as muscimol also have anxiolytic-like effects in animal models of anxiety, particularly in the septum (e.g., Drugan, Skolnick, Paul, & Crawley, 1986). Thus one purpose of the present studies was to examine the effects of intra-septal infusions of muscimol on rats' fear behaviours in the plus-maze and shock-probe tests.

With respect to the hippocampus, there is evidence that hippocampal cholinergic systems may be particularly involved in the modulation of anxiety. For example, increases in rats' fear reactions have been observed in a variety of tests following intra-hippocampal infusions of both muscarinic and nicotinic antagonists (File, Gonzalez, & Andrews, 1998; Hess & Blozovski, 1987; Smythe, Murphy, Bhatnagar, Timothy, & Costall,1998). One expectation, based on these antagonist studies, is that a general up-regulation of hippocampal cholinergic systems might result in anxiety-reduction. Thus the second purpose of the present study was to examine the effects of intra-hippocampal infusions of the acetylcholinesterase inhibitor physostigmine on rats' fear responses. Finally, it is possible that septal GABAergic systems might interact with hippocampal cholinergic systems in the control of anxiety. Thus, the independent and combined effects of stimulating septal GABAergic systems and hippocampal cholinergic systems was examined.

Anxiety was assessed in the elevated plus-maze and the shock-probe burying tests. In the elevated plus-maze test, rats typically avoid the open arms of the maze and spend most of their time in the two enclosed arms (Pellow, Chopin, File, & Briley, 1985). In the shock-probe burying test, rats shocked from a stationary, electrified probe push bedding material from the floor of the experimental chamber toward the shock-probe (i.e., burying) while avoiding further contacts with the probe (Pinel & Treit, 1978; Treit, Pinel, & Fibiger, 1981; Treit, Menard, & Pesold, 1994). Anxiolytic drugs such as diazepam increase open-arm exploration in the plus-maze and decrease burying toward the shock-probe (De Boer, Slangen, & Van der Gugten, 1990; Pellow, 1986; Pellow et al., 1985; Pellow & File, 1986; Treit et al., 1981; Treit, Pesold, & Rotzinger, 1993b; Treit et al., 1994; Tsuda, Yoshishige, & Tanaka, 1988). Conversely, anxiogenic drugs such as yohimbine, decrease open-arm exploration while increasing shock-probe burying (Johnston & File, 1989; Pellow, 1986; Pellow et al., 1985; Pellow & File, 1986; Treit, 1990; Tsuda et al., 1988). Thus, anxiolytic effects in shock-probe and plus-maze tests involve a decrease and an increase in activity, respectively. This suggests that reductions in anxiety in both tests would be difficult to explain in terms of non-specific effects on general activity, arousal, pain sensitivity, or behavioral inhibition. Furthermore, neither test involves response learning or an explicit memory requirement, factors that can complicate the interpretation of drug effects in other animal models of anxiety (e.g., the conflict test; Treit, 1985). The combined use of rats' untrained fear reactions in two different pharmacologically validated tests of anxiety is particularly important in the present study, since both septal GABAergic and hippocampal cholinergic systems have been repeatedly implicated in learning and memory (e.g., Brioni, Decker, Gamboa, Izquierdo, & McGaugh, 1990; Degroot and Parent, 2000; Durkin, 1992).

2. Experiment 1

2.1. Materials and Methods

2.1.1. Subjects

The subjects were naive, male albino Sprague-Dawley rats, purchased from Charles River, Canada, weighing 300-350 g at the time of surgery. Following surgery, rats were individually housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle (lights on at 07.00h), with food and water available ad libitum. Behavioral testing occurred between 09.00 and 19.00 h. The treatment of all animals was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.1.2. Surgery and Histology

The rats were given an oral administration of the analgesic acetaminophen (Tylenol 120 mg/1.5 ml) followed one hour later by atropine sulfate (0.1 mg/0.2 ml, i.p.) to reduce respiratory complications due to the anaesthetic. The rats were then anaesthetized with pentobarbitol (Nembutal 50 mg/kg, i.p.), hydrated with saline (3 cc, s.c.), and given the antibiotic penicillin (Crystiben, Rhone Merieux Canada Inc., 15,000 I.U./0.05 cc, i.m.). Stereotaxic procedures were used to implant 45 rats with one 22 gauge stainless-steel guide cannula (Plastics One, Inc. Roanoke, VA) aimed at the medial septum (0.5 mm anterior to bregma [AP], 4.9 mm ventral to dura [DV], using flat skull coordinates (Paxinos and Watson, 1986) and 45 rats with two 22 gauge cannulae aimed bilaterally at the lateral septum (0.7 mm AP, 3.0 mm lateral to bregma, 4.6 mm DV, with the cannula angled 22° medially. The cannulae were attached to the skull with 4 jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula to keep the cannula tract clear. Immediately after surgery the rats were placed into a warm environment until they regained consciousness. Two days after surgery, each cannula was checked for obstructions, and betadine was applied to the surgical wound. Following behavioral testing, rats were sacrificed with an overdose of chloral hydrate and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and placed in 10% formalin solution. After at least 48 h had elapsed, the brains were frozen with dry ice, sectioned (60 μm), mounted onto glass slides and stained with thionin. The location of the cannulae for each rat was examined microscopically by an observer who was "blind" to the behavioral results. The location of the cannulae tips were then transcribed onto the appropriate atlas plates (Paxinos and Watson, 1986). The behavioral data for animals with necrosis of brain tissue at the site of implantation (n = 1) or misplaced cannulae (n = 13) were discarded (n = 14). It is important to note that any cannula tip (bilateral or unilateral) observed outside of its intended target was deemed "misplaced." In addition, one rat failed to contact the probe in the shock-probe burying test and therefore was not included in the analyses of the burying data.

2.1.3. Infusion procedures

Rats in each of the two surgical groups were randomly assigned to one of the following two drug conditions: (1) a control condition, infused with vehicle or (2) a drug condition, infused with muscimol. The tame, hand-held rats were given an infusion of muscimol (20 ng/0.4 μl/1 min) or its vehicle (phosphate buffered saline; PBS, pH 7.4; 0.4 μl/1 min) into the medial or lateral septum through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula. The internal cannula was connected to a 10 μl constant rate Hamilton microsyringe with polyethylene tubing and the infusions were delivered using an infusion pump (Harvard Apparatus 22). The internal cannula was left in place for 1 min following the infusions in order to allow for diffusion. The muscimol dose was selected based on pilot work in our laboratory.

2.1.4. Behavioral Testing

The behavioral testing procedures were the same as those used in previous experiments (e.g., Menard and Treit, 2000, Pesold and Treit, 1996; Treit et al., 1993b, 1994). All behavior was recorded on videotape for ensuing analysis. Plus-maze testing occurred at least 7 days post-surgery and shock-probe testing occurred at least 12 days post-surgery. This order of testing is based on earlier work (Treit and pesold, 1990) showing no effect of a 5-min exposure to the plus-maze on subsequent behavior in the shock-probe test, but some disruption of plus-maze behavior when preceded by a shock-probe test. Drug conditions (vehicle versus muscimol) were counterbalanced across the two behavioral tests.

2.1.5. Plus-maze

This wooden, plus-shaped apparatus was elevated to a height of 50 cm, and consisted of two 50 x 10 cm open arms, and two 50 x 10 x 50 cm enclosed arms, each with an open roof. The maze was in the center of a quiet and dimly lit room. The rats' behavior was observed using a mirror that was suspended at an angle above the maze. Behavioral data were collected by a "blind" observer who quietly sat one meter behind one of the closed arms of the maze. Five minutes following their respective drug treatment, rats were placed individually in the center of the plus-maze, facing one of the closed arms. The observer measured (1) time spent in the open arms, (2) time spent in the closed arms, (3) number of entries into the open arms, and (4) number of entries into the closed arms, during the five-minute test period. An entry was defined as all four paws in the arm. The maze was cleaned with distilled water after each rat was tested. For the purpose of analysis (Pellow et al, 1985; Pellow & File, 1986), open-arm activity was quantified as the amount of time that the rat spent in the open arms relative to the total amount of time spent in any arm (open/total x 100), and the number of entries into the open arms was quantified relative to the total number of entries into any arm (open/total x 100). The total number of arms entered was used as an index of general activity (for details see Pellow et al., 1985; Rodgers and Johnson, 1995).

2.1.6. Shock-probe

The shock-probe burying apparatus consisted of a 40 x 30 x 40 cm plexiglass chamber, evenly covered with approximately 5 cm of bedding material (odor-absorbent kitty litter). The shock-probe was inserted through a small hole on one wall of the chamber, 2 cm above the bedding material. The plexiglass shock-probe (6.5 cm long and 0.5 cm in diameter) was helically wrapped with two copper wires through which an electric current was administered. Current from the 2000 V shock source (60 cycle AC RMS) was varied with potentiometers and set at 2 mA. Rats were habituated in pairs to the test chamber without the shock-probe, for 30 minutes on each of four consecutive days prior to the test day. On the test day, five minutes following their respective drug treatments, rats were individually placed in one corner of the testing chamber, facing away from the shock-probe. The probe was not electrified until the rat touched it with its snout or forepaws, at which point the rat received a brief, 2 mA shock. The 15-minute testing period began once the rat received its first shock and the probe remained electrified for the remainder of the testing period. Following the first shock, the duration of time each rat spent spraying bedding material toward or over the probe with its snout or forepaws (i.e. burying behavior) was measured, as was the total number of contact-induced shocks each rat received from the probe. An index of the rat's reactivity to each shock was scored according to the following four-point scale (Pesold and Treit, 1992): (1) flinch involving only head or forepaw, (2) whole body flinch, with or without slow ambulation away from the probe, (3) whole body flinch, and/or jumping, followed by immediate ambulation away from the probe, and (4) whole body flinch and jump (all four paws in the air), followed by immediate and rapid ambulation (i.e. running) to the opposite end of the chamber. A mean shock reactivity score was calculated for each rat by summing its shock reactivity scores and dividing by the total number of shocks it received. The total time that the rat spent immobile (e.g. resting on the floor) during the 15-minute testing period was used as an index of general activity. All behavior measures were made by a "blind" observer who was watching the rat via a video monitor in a room adjacent to the testing room.

2.1.7. Statistical Analysis

Results are expressed as means and standard errors of the mean (S.E.M.). The plus-maze data and the shock-probe data were analyzed using analysis of variance (ANOVA). In order to correct for non-normality and heterogeneity of variance, the burying scores were transformed to their square roots prior to ANOVA. Measures of anxiety (i.e., open-arm exploration and shock-probe burying) were analyzed by *a priori* F tests between the muscimol drug groups and their respective vehicle controls. Control measures (e.g., shock-reactivity, total arm entries) were analyzed by overall between-groups ANOVAs (alpha = 0.05).

2.2. Results

2.2.1. Plus-maze

Fig. 4-1 shows the location of the infusion sites in the medial and lateral septum. Fig. 4-2 indicates that muscimol infusions into either the medial or lateral septum produced clear anxiolytic-like effects in the plus-maze. Specifically, rats infused with 20 ng of muscimol into the medial septum showed a significantly greater percentage of open-arm entries [$\underline{F}(1,34) = 8.19$, $\underline{p} = 0.01$] and open-arm time [$\underline{F}(1,34) = 4.11$; $\underline{p} = 0.05$] than their vehicle-infused controls. Similarly, rats infused with 20 ng of muscimol into the lateral septum displayed a significantly greater percentage of open arm entries [$\underline{F}(1,34) = 6.91$; $\underline{p} = 0.01$] and open-arm time [$\underline{F}(1,34) = 5.90$; $\underline{p} = 0.02$] compared to their vehicle infused controls. There was no indication of non-specific changes in general activity, as the total number of arms entered did not differ between groups [$\underline{F}(3,68) = 0.77$; $\underline{p} = 0.51$; see table 1]. Although the number of closed arms entered did differ significantly between groups [$F(3, 68) = 3.54$; $p = 0.02$;

see table 4-1], *post hoc* comparisons (Bonferroni test, alpha = 0.05) revealed no significant differences between rats infused with muscimol in the medial or lateral septum and their respective vehicle-infused controls.

2.2.2. Shock-probe

Figure 4-3 indicates that muscimol infusions also produced anxiolytic-like effects in the shock probe burying test. Rats infused with 20 ng of muscimol in the medial septum [$\underline{F}(1,34) = 8.04$, $\underline{p} = 0.01$] or the lateral septum [$\underline{F}(1,34) = 4.85$, $\underline{p} = 0.03$] each displayed significantly lower burying levels than their respective vehicle-infused controls. This anxiolytic-like effect occurred in the absence of any significant changes in immobility [$\underline{F}(3,68) = 0.68$, $\underline{p} = 0.57$], number of shocks received [$\underline{F}(3,68) = 0.57$, $\underline{p} = 0.64$], or shock reactivity [$\underline{F}(3,68) = 0.47$, $\underline{p} = 0.70$; see table 4-2]. Taken together, these data suggest stimulating GABAergic receptors with a 20 ng dose of muscimol, in either the medial or lateral septum, produces behaviorally specific anxiolytic-like effects in both the plus-maze and shock-probe burying tests.

3. Experiment 2

Experiment 2 examined the individual and combined effects of stimulating the septal GABAergic and hippocampal cholinergic systems. Based on the anxiogenic effects previously found after intra-hippocampal administration of cholinergic antagonists (see introduction), we expected that up-regulation of hippocampal cholinergic systems should have anxiolytic-like effects, possibly equivalent to those of intra-septal GABAergic receptor stimulation. In addition, given the extensive interconnections of the hippocampus and the septum, it seemed possible that an interaction could arise from the simultaneous stimulation of the two systems. In order to examine these hypotheses, rats were implanted with guide cannulae in both the medial septum and the dorsal hippocampus and infused with muscimol in the septum and physostigmine in the hippocampus.

3.1. Materials and Methods

The methods and procedures used in this experiment were basically the same as those used in the first two experiments. Stereotaxic procedures were used to implant 120 rats with one 22 gauge cannula aimed at the medial septum and one 22 gauge cannula aimed at the dorsal hippocampus (-4.2 mm AP, 2.0 mm DV, 4.1 mm lateral to the midline). We decided to implant guide cannulae in the medial septum based on the positive results found in Experiment 1 and because it was surgically more convenient. Half of the rats (n = 60) had the hippocampal cannulae implanted in the left hemisphere. We chose to examine the effects of unilateral hippocampal infusions for surgical convenience, and because it has been demonstrated that perfusions into one hippocampal hemisphere affects acetylcholine in both hippocampal hemispheres (Ragozzino et al., 1998). The behavioral data for animals with necrosis of brain tissue at the site of implantation (N = 25) or misplaced cannulae (n = 23) were discarded. "Misplacements" occurred when either one or both of the two cannulae were outside of their intended targets. The tame, hand-held rats were given an infusion of muscimol (20 ng/0.4 ul/1 min) or vehicle (PBS; 0.4 ul/1 min) into the medial septum through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula, or an infusion of physostigmine (10 µg/1 µl/1 min) or its vehicle (PBS; 1 µl/1 min) into the dorsal hippocampus through a 26 gauge stainless steel internal cannula lowered 0.8 mm below the tip of the guide cannula. The infusions were delivered using an infusion pump (Harvard Apparatus 22). The 20 ng dose of muscimol was selected based on the findings from Experiment 1. The 10 μg dose for physostigmine was based on previous results (Degroot and Parent, 2000). Rats were randomly assigned to one of the four drug conditions: (1) vehicle in both the hippocampus and the medial septum (Veh-Veh), (2) physostigmine in the hippocampus and vehicle in the medial septum (Phys-Veh), (3) vehicle in the hippocampus and muscimol in the medial septum (Veh-Mus), or (4) physostigmine in the hippocampus and muscimol in the medial septum (Phys-Mus).

3.1.1. Statistical Analysis

Results are expressed as means and standard errors of the mean (S.E.M.). The plus-maze data and the shock-probe data were analyzed using analysis of variance (ANOVA). As for Experiment 1, in order to correct for non-normality and heterogeneity of variance, the burying scores were transformed to their square roots prior to ANOVA. Measures of anxiety (i.e., open-arm exploration and shock-probe burying) were analyzed by *a priori* F tests between the muscimol drug groups and their respective vehicle controls. Control measures (e.g., shock-reactivity, total arm entries) were analyzed by overall between-groups ANOVAs ($\alpha = 0.05$).

3.2. Results

3.2.1. Plus-maze

Figs. 4-4 and 4-5 show the location of the infusion sites in the medial septum and the hippocampus, respectively. Fig. 4-6 shows that all three drug conditions (Phys-Veh, Veh-Mus, Phys-Mus) produced an increase in the percentage of open arm entries (panel A) and open arm time (panel B) compared to the Veh-Veh control condition. Concurrent infusions of physostigmine in the hippocampus and vehicle in the medial septum (Phys-Veh) increased the percentage of entries into the open arms [$F(1,37) = 6.40$, $p = 0.02$] and the percentage of time spent in the open arms [$F(1,37) = 7.57$, $p = 0.01$] compared to Veh-Veh controls. Veh-Mus rats also demonstrated significantly more open-arm entries [$F(1,35) = 7.67$, $p = 0.01$] and open-arm time [$F(1,35) = 4.15$, $p = 0.05$] compared to Veh-Veh controls Similarly, Phys-Mus rats entered significantly more open arms than did Veh-Veh rats [$F(1,30) = 4.64$, $p = 0.04$], although their time spent on the open arms failed to reach significance [$F(1,30) = 2.04$, $p = 0.16$]. Neither the total number of arms entered [$F(3, 68) = 0.22$, $p = .88$; see Table 4-2] nor the number of closed arms entered [$F(3, 68) = 1.49$, $p = .23$; see Table 4-2] differed significantly between the groups.

3.2.2. Shock-probe

Fig. 4-7 shows a large anxiolytic effect in all three drug groups in comparison to Veh-Veh control. Statistical analysis confirmed that all drug groups buried the shock-probe at significantly lower levels than Veh-Veh controls [Phys-Veh: $F(1,35) = 8.07$, $p = 0.01$; Veh-Mus: $F(1,34) = 9.64$, $p = 0.01$; Phys-Mus: $F(1,31) = 6.59$, $p = 0.02$]. These anxiolytic effects were not confounded by between-group differences in general activity [$F(3,67) = 2.44$, $p = 0.07$], or number of shocks received [$F(3,67) = 1.12$, $p = 0.35$]. Although there was a significant between-group difference in shock-reactivity [$F(3,67) = 5.08$, $p = 0.01$; see table 4-3], subsequent correlational analysis showed there was no systematic relationship between rats' shock-reactivity scores and their burying scores (pearson's $r = 0.07$, $p = 0.5$).

4. General Discussion

The present results indicate that increasing ACh levels in the hippocampus or stimulating GABAergic receptors in the medial or the lateral septum decreases anxiety as measured in the elevated plus-maze and the shock-probe burying tests. Moreover, the data suggest that stimulating hippocampal cholinergic and septal GABAergic receptors simultaneously does not perturb the anxiolytic effect obtained by stimulating either receptor system independently. These anxiolytic-like effects are difficult to explain in terms of non-specific drug effects on general activity, arousal, or behavioral inhibition since anxiety-reduction in the plus-maze is indicated by a selective increase in a specific activity while anxiety-reduction in shock-probe is indicated by a selective decrease in a specific activity. All rats avoided the shock-probe to a similar extent, suggesting they were quite capable of associating the shock with the probe. Furthermore, both tests utilize rats' untrained fear reactions to clear and present anxiogenic stimuli, making interpretations in terms of learning or memory processes even less likely. In one instance rats did differ in their reactivity to the shock-probe, but this effect was not correlated with burying behavior. Thus, our study may be the first to provide clear evidence that increasing hippocampal ACh levels results in a decrease in anxiety. In addition, the study confirmed that stimulating septal GABAergic receptors with a directly acting $GABA_A$ agonist, muscimol, can have effects that are nearly indistinguishable from traditional benzodiazepine anxiolytics, which are indirect modulators of the $GABA_A$ receptor site.

It was our original intention to demonstrate site specificity by comparing data obtained from rats with misplaced cannulae with that from rats whose cannulae were correctly placed. However, in Experiment 1, animals whose cannulae were placed outside of the intended target area (either medial or lateral septum) still received a (unilateral) infusion within the septal area. Since one of the targets (medial septum or lateral septum) was very likely stimulated, comparisons between “hits” and “complete misses” could not be made. Furthermore, in Experiment 2, at least one cannula was always located within the intended target area. Therefore, in the case of combined drug infusions, one of the target areas (hippocampal cholinergic or septal GABAergic receptors) was likely stimulated. In the case of single infusions, “misplaced” cannulae were still not totally outside the target region. This problem prevented unambiguous comparisons of “hits” and “misses.”

The anxiolytic effects induced by stimulating the hippocampal cholinergic system are consistent with previous data which indicate that intra-hippocampal infusions of cholinergic antagonists increase anxiety (File, Gonzalez, & Andrews, 1998b; Hess and Blozovski, 1987; Smythe et al., 1998). However, our data are more difficult to reconcile with previous studies of the effects of intra-hippocampal infusions of cholinergic agonists such as nicotine, which has been found to be anxiolytic, anxiogenic, or without effect in animal models of anxiety (File, Kenny, & Ougazzal, 1998a; Ouagazzal, Kenny, & File, 1999). Some of these inconsistencies may be due to differences between animal testing procedures, differences in basal “anxiety” levels, or to differences in the relative specificity of cholinergic manipulations. In our study it is likely that all cholinergic receptor sub-types would have been stimulated simultaneously, which resulted in a net anxiolytic effect. To investigate this issue further, it might be useful to co-infuse intra-hippocampal physostigmine with specific cholinergic receptor antagonists. Receptor-specific antagonism of the effects of physostigmine would more clearly characterize the neurochemical substrates of anxiety in the hippocampus.

The similarity of the anxiolytic effects of muscimol infused into the lateral and medial septum is consistent with lesion studies (e.g., Menard & Treit, 1996b), but contrasts with a study using intra-septal infusion of the benzodiazepine anxiolytic midazolam, where a dissociation was found (Pesold & Treit, 1996). In the latter study, anxiolytic effects were found only when midazolam was infused into the lateral septum, not the medial septum. It is possible that muscimol, because it is a directly acting $GABA_A$ agonist, is more potent than midazolam, and thereby more likely to display anxiolytic effects in both septal areas. Diffusion of msucimol from the medial septum to the lateral septum seems unlikely since we used a small injection volume (0.4 μl).

It seems curious that simultaneous stimulation of both hippocampal cholinergic and septal GABAergic receptors did not result in a larger anxiolytic effect, compared to the independent stimulation of each of these receptor systems. One possibility is that separate stimulation of each of these systems already produced maximal anxiolytic effects in both tests (i.e., nearly 50% open-arm exploration in the plus-maze test and a substantial suppression of burying in the shock-probe test). Thus, these behavioral "ceiling" and "floor" effects could have masked any further reduction in anxiety that might otherwise have been observed when both cholinergic and GABAergic systems were simultaneously stimulated. It is also possible that the independent stimulation of each receptor system in our study resulted in asymptotic receptor activation. Thus, a further stimulation of either or both systems would not result in a further reduction in anxiety. For these reasons it may be premature to conclude that the hippocampal cholinergic and septal GABAergic receptor systems do not act in concert in the control of anxiety. Studies in which sub-threshold doses of muscimol and physostigmine are infused into the septum and hippocampus, respectively, might indicate whether and how the two systems cooperate in the control of anxiety.

It is unlikely that the hippocampus influenced anxiety via an interaction with the amygdala, a structure which is also involved in fear and anxiety (e.g., Davis, 1992; LeDoux, 1990). Although the hippocampus is extensively connected to the amygdala (e.g. Pikkarainen, Ronkko, Savander, Insausti, & Pitkanen, 1999), there is little evidence that the amygdala regulates open-arm exploration in the plus-maze or burying behavior in shock-probe tests (Pesold & Treit, 1994; Treit & Menard, 1997; Treit et al., 1993b; but see Pesold and Treit, 1995).

In summary, the present findings demonstrate that stimulating the GABAergic system of the medial or the lateral septum as well as increasing hippocampal acetylcholine levels reduces anxiety. Stimulating hippocampal cholinergic and septal GABAergic receptors simultaneously does not increase the anxiolytic effect obtained by stimulating either receptor system independently.

Table 4-1. Mean (+/- S.E.M.) total arm entries in the plus-maze task, and mean (+/- S.E.M.) activity and reactivity in the shock-probe burying test.

Plus-maze

	Medial Septum		Lateral Septum	
	Vehicle (n = 18)	Muscimol (n = 18)	Vehicle (n = 17)	Muscimol (n = 19)
Total arm entries	11.83	11.28	12.18	10.53
	(0.88)	(0.81)	(0.82)	(0.78)
Closed arm entries	8.39	6.39	8.94	6.21
	(0.66)	(0.84)	(0.70)	(0.72)

Shock-probe

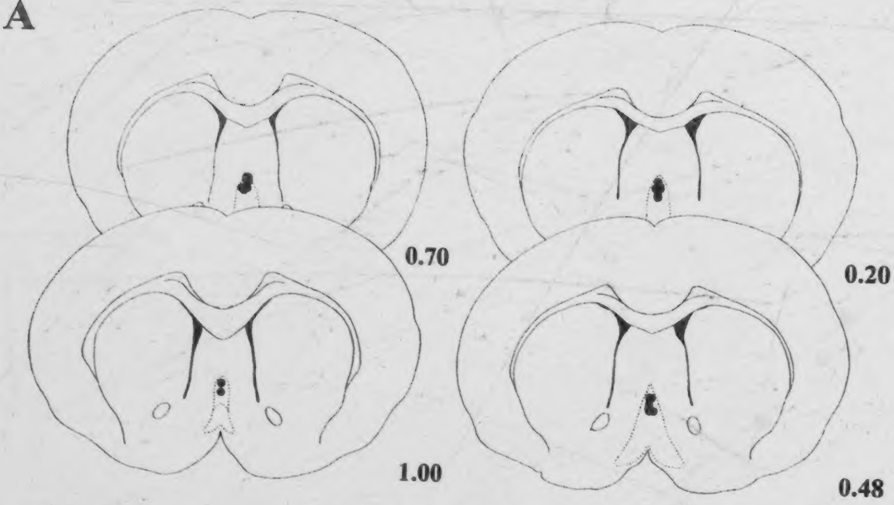
	(n = 17)	(n = 19)	(n = 19)	(n = 17)
Immobility (s)	51.41	98.95	52.05	49.24
	(36.35)	(37.93)	(21.57)	(16.18)
Shock Reactivity	2.29	2.16	2.22	2.41
	(0.15)	(0.15)	(0.17)	(0.16)
Shock number	1.59	1.68	1.63	1.94
	(0.21)	(0.15)	(0.19)	(0.26)

Table 4-2. Mean (+/- S.E.M.) total arm entries in the plus-maze task, and mean (+/- S.E.M..) activity and reactivity in the shock-probe burying test.

		Plus-maze		
	PBS/PBS (n = 18)	PHYS/PBS (n = 21)	PBS/MUSC (n = 19)	PHYS/MUSC (n = 14)
Total arm entries	12.39	12.76	12.63	11.64
	(1.05)	(1.02)	(0.96)	(1.03)
Closed arm entries	10.22	8.33	7.74	8.29
	(0.91)	(0.88)	(0.89)	(0.85)
		Shock-probe		
	(n = 17)	(n = 20)	(n = 19)	(n = 16)
Immobility (s)	57.41	63.35	117.53	175.81
	(16.07)	(15.35)	(44.24)	(49.81)
Shock reactivity	2.08	1.73	2.09	2.30
	(0.10)	(0.12)	(0.13)	(0.14)
Shock number	2.06	2.10	1.63	2.19
	(0.30)	(0.28)	(0.17)	(0.25)

Figure 4-1. Schematic illustration of coronal sections of the rat brain showing the approximate location of A) medial septal and B) lateral septal infusion sites in Experiment 1. The numbers indicate sections anterior to Bregma. Atlas plates adapted from Paxinos and Watson (1986).

A


0.70
0.20
1.00
0.48

B

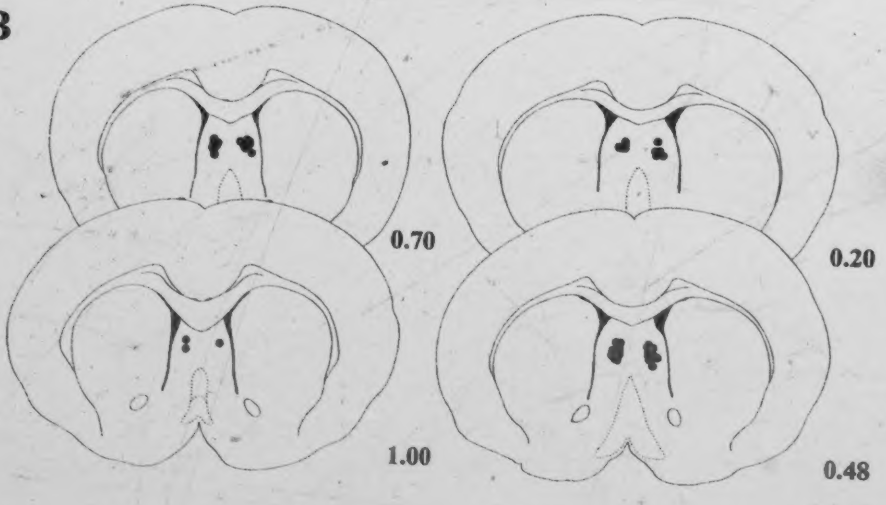

0.70
0.20
1.00
0.48

Figure 4-2. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of muscimol (20 ng/0.4 μl) or vehicle (0.4 μl) into the medial or lateral septum. *p < .05 compared with the vehicle control group.

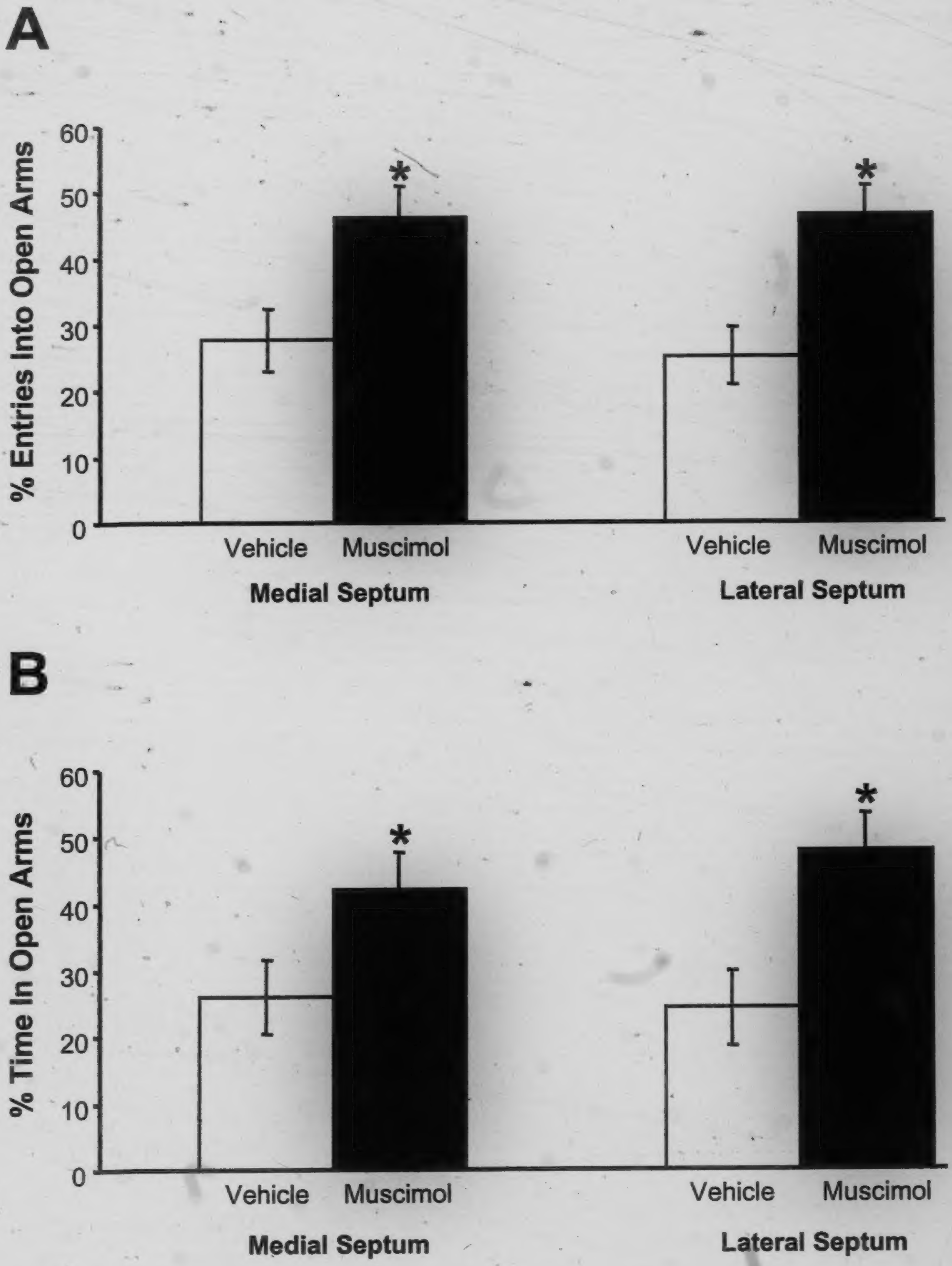
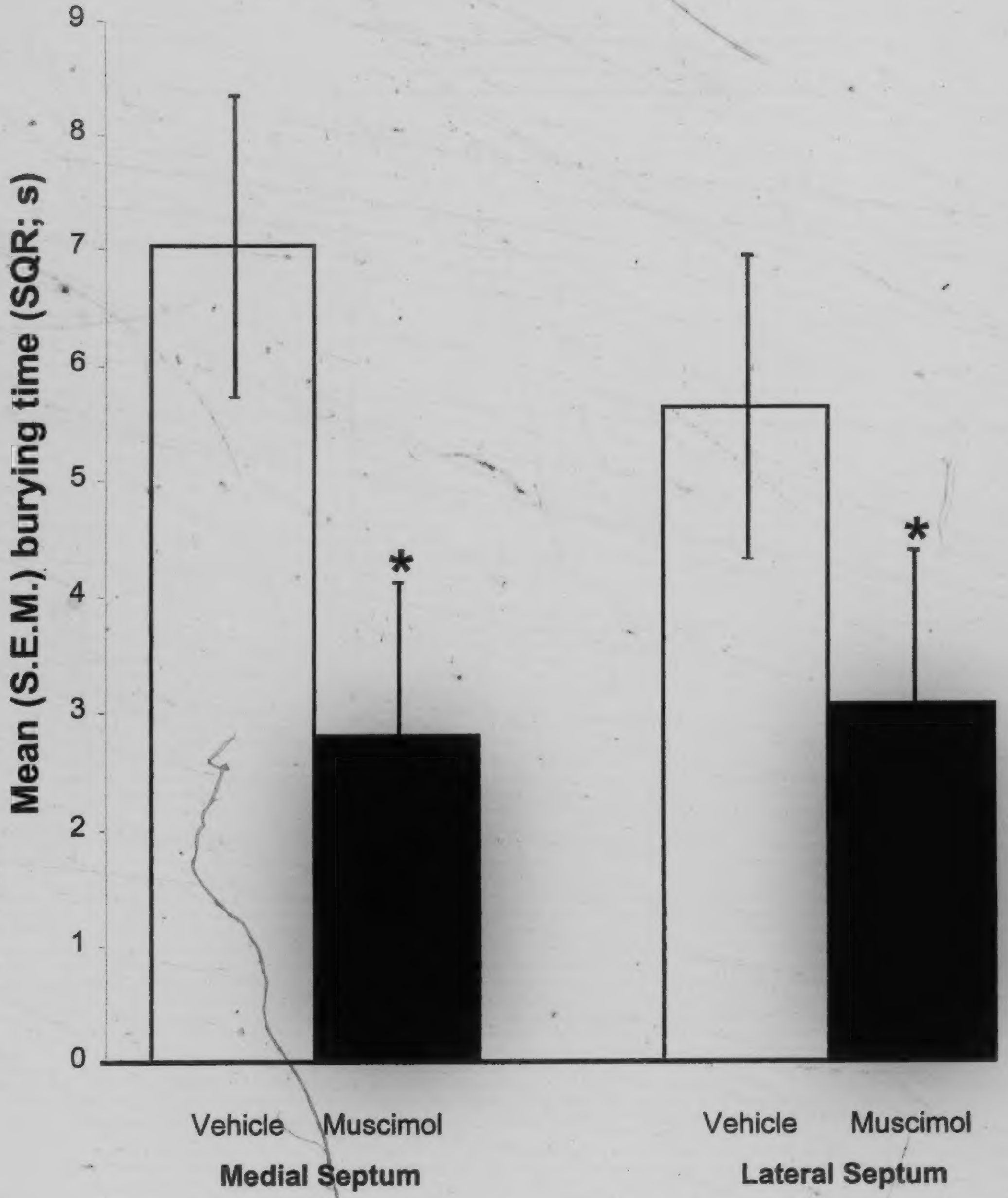

A
% Entries Into Open Arms
0
10
20
30
40
50
60
*
*
Vehicle
Muscimol
Medial Septum
Vehicle
Muscimol
Lateral Septum
B
% Time In Open Arms
0
10
20
30
40
50
60
*
*
Vehicle
Muscimol
Medial Septum
Vehicle
Muscimol
Lateral Septum

Figure 4-3. Mean (+/- S.E.M.) bury time (SQR; s) in the shock probe apparatus after infusions of muscimol (20 ng/0.4 µl) or vehicle (0.4 µl) into the medial or lateral septum. *p < .05 compared with the vehicle control group.



Mean (S.E.M.) burying time (SQR; s)
9
8
7
6
5
4
3
2
1
0
*
*
Vehicle
Muscimol
Medial Septum
Vehicle
Muscimol
Lateral Septum

Figure 4-4. Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septal infusion sites in Experiment 3. The numbers indicate sections anterior to Bregma. Atlas plates adapted from Paxinos and Watson (1986).

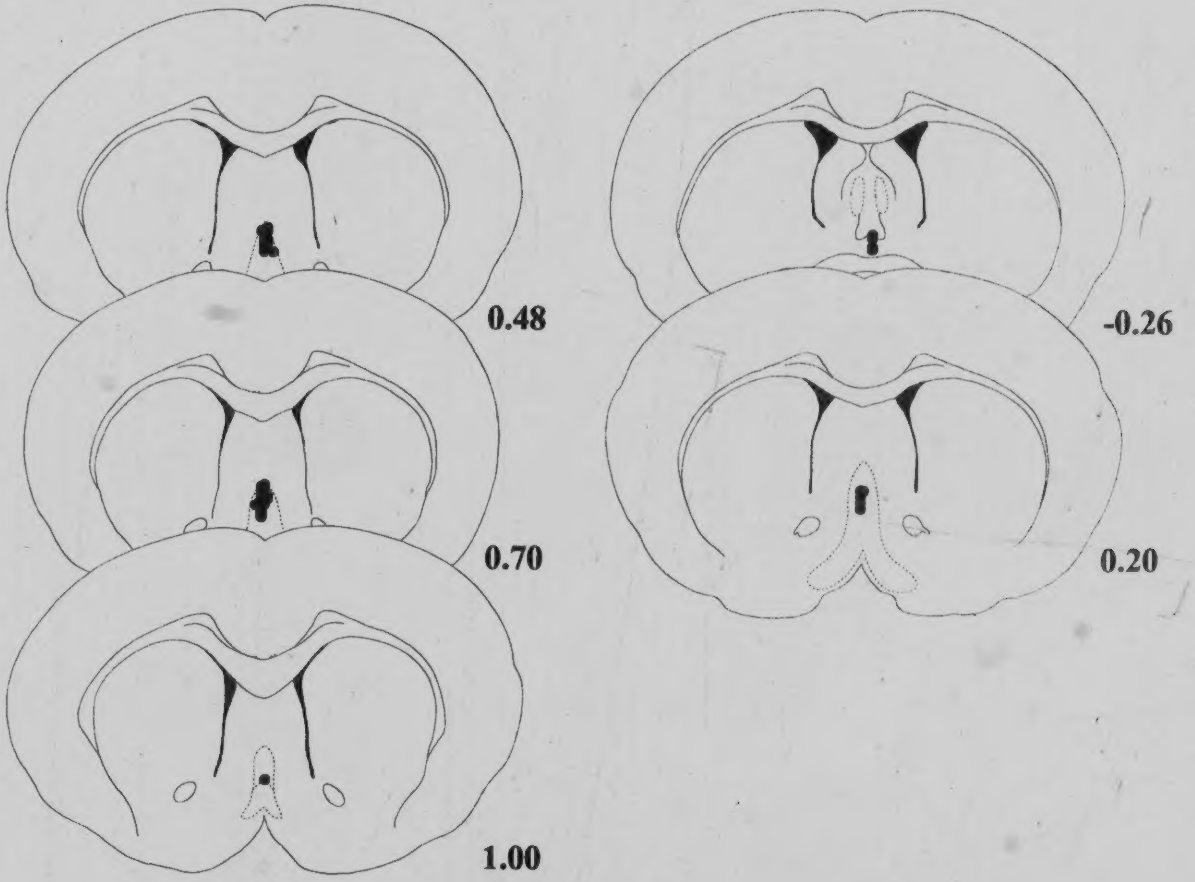

0.48
0.70
1.00
-0.26
0.20

Figure 4-5. Schematic illustration of coronal sections of the rat brain showing the approximate location of hippocampal infusion sites in Experiment 3. The numbers indicate sections posterior to Bregma. Atlas plates adapted from Paxinos and Watson (1986).

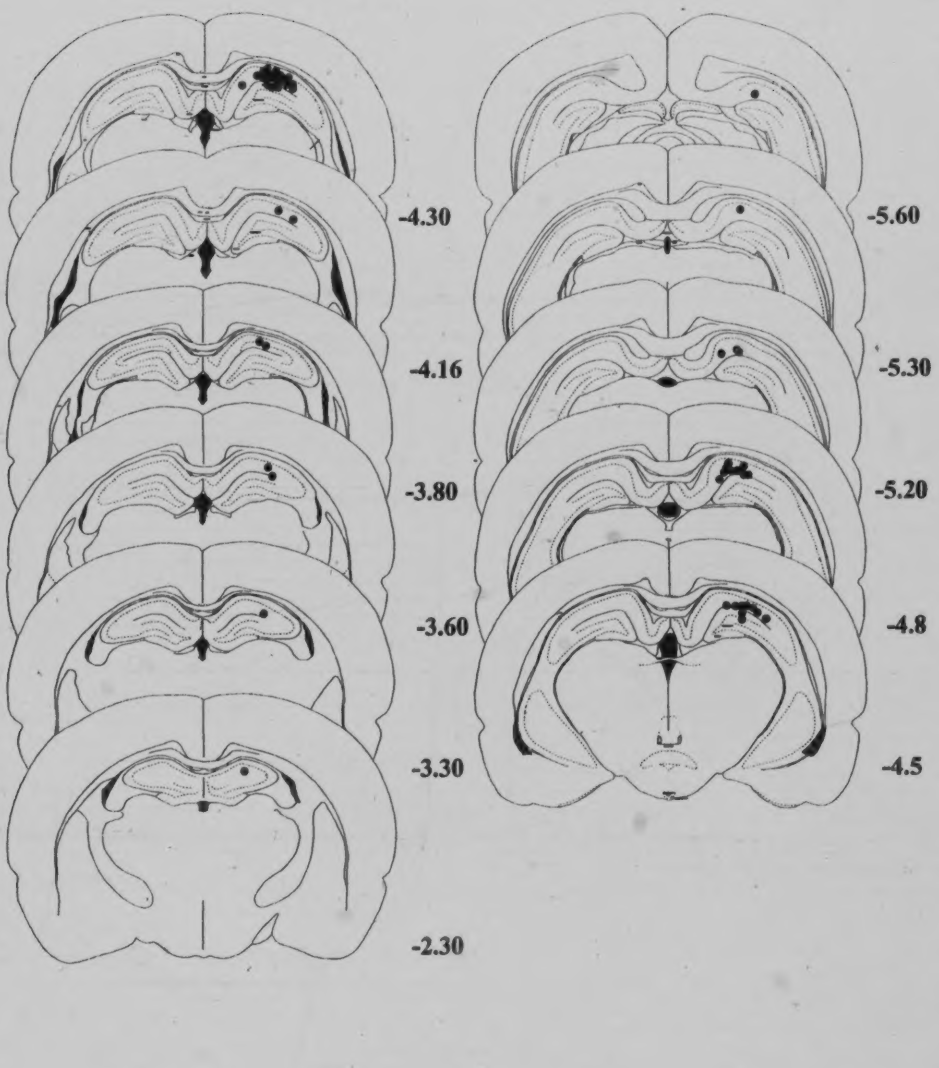

-4.30
-4.16
-3.80
-3.60
-3.30
-2.30
-5.60
-5.30
-5.20
-4.8
-4.5

Figure 4-6. Mean (+/- S.E.M.) percent open arm entries (A) and percent open arm time (B) in the elevated plus maze after infusions of muscimol (20 ng/0.4 µl) into the medial septum, physostigmine (10 µg/1 µl) into the dorsal hippocampus, or combined infusions. *p < .05 compared with the vehicle control group.

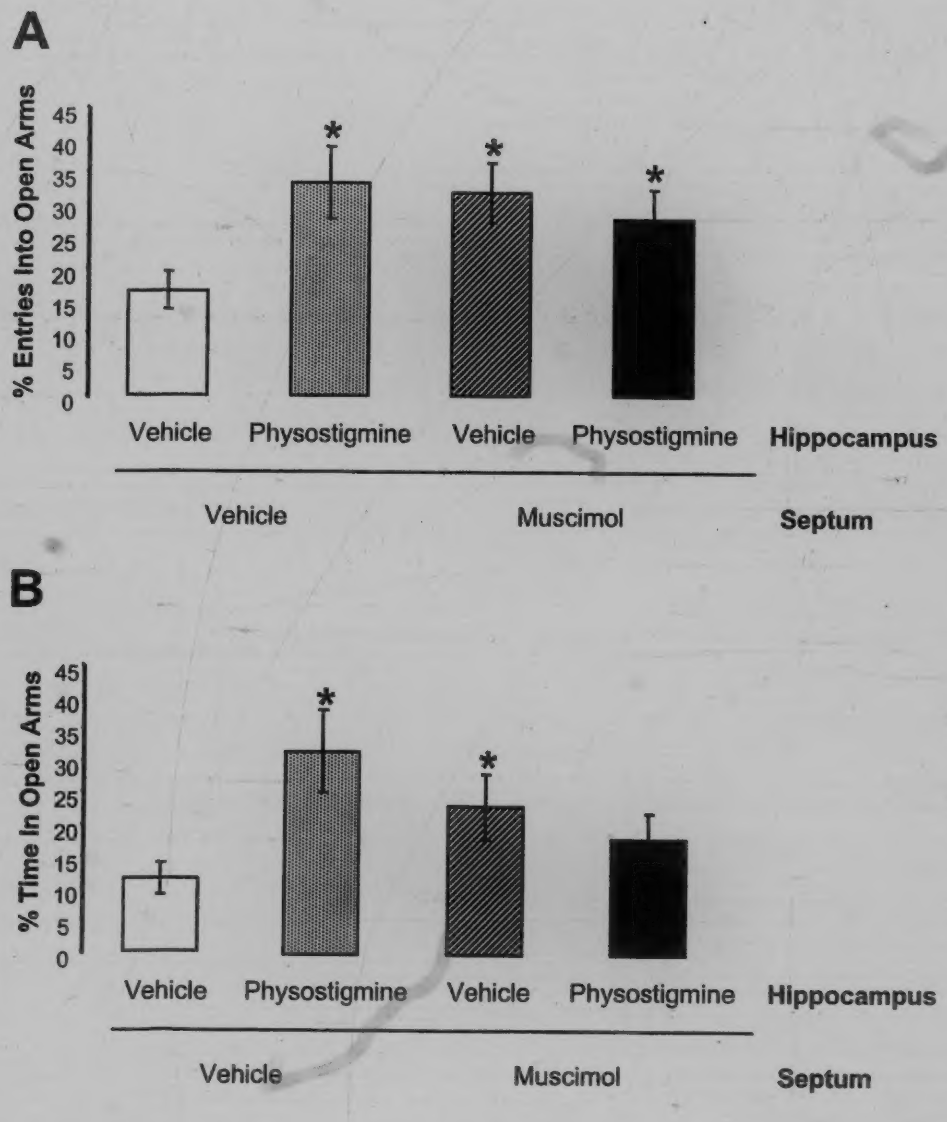
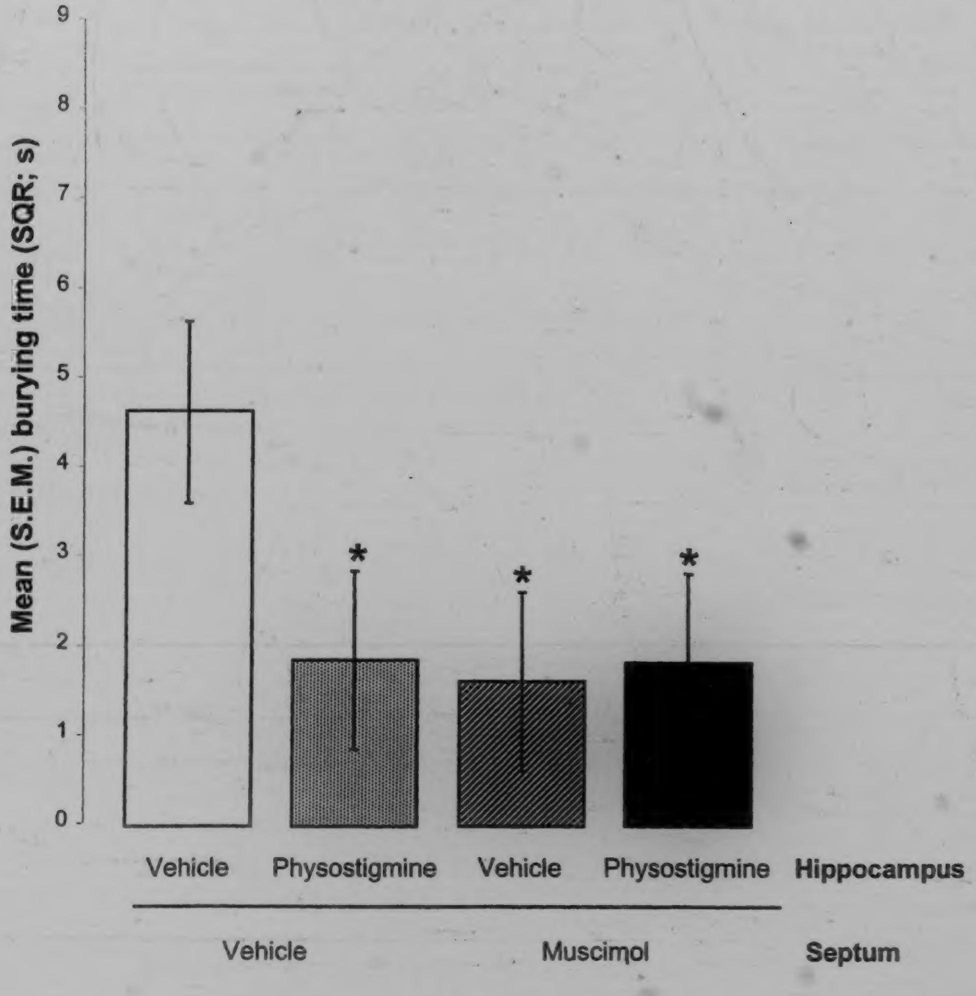

A
% Entries Into Open Arms
45
40
35
30
25
20
15
10
5
0
*
*
*
Vehicle
Physostigmine
Vehicle
Physostigmine
Hippocampus
Vehicle
Muscimol
Septum
B
% Time In Open Arms
45
40
35
30
25
20
15
10
5
0
*
*
Vehicle
Physostigmine
Vehicle
Physostigmine
Hippocampus
Vehicle
Muscimol
Septum

Figure 4-7. Mean (+/- S.E.M.) bury time (SQR; s) in the shock probe apparatus after infusions of muscimol (20 ng/0.4 μl) into the medial septum, physostigmine (10 ng/1 μl) into the dorsal hippocampus, or combined infusions. *p < .05 compared with the vehicle control group.


Mean (S.E.M.) burying time (SQR; s)
0
1
2
3
4
5
6
7
8
9
*
*
*
Vehicle
Physostigmine
Vehicle
Physostigmine
Hippocampus
Vehicle
Muscimol
Septum

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Chapter 5

Septal GABAergic and Hippocampal Cholinergic Systems Interact in the Modulation of Anxiety.

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Extensive evidence has implicated the septum in fear and anxiety. For example, we have repeatedly found that ablation or pharmacological inhibition of this area reduces rats' untrained fear reactions, suggesting that the septum normally plays an excitatory role in the control of anxiety (for reviews see; Menard and Treit, 1999; Treit and Menard, 2000). Specifically, we found that electrolytic or excitotoxic lesions of the septum produced anxiolytic-like effects in the plus-maze and shock-probe burying tests, i.e., open-arm exploration was increased and burying behavior was decreased (Treit and Pesold, 1990; Pesold and Treit, 1992; Treit et al., 1993a; Menard and Treit, 1996a,b). These anxiolytic effects were also produced when septal activity was inhibited via intra-septal infusions of the benzodiazepine-type anxiolytic, midazolam, an indirect $GABA_A$ agonist (Pesold and Treit, 1994, 1996) or muscimol, a direct $GABA_A$ agonist (Degroot et al., 2001).

Likewise, the hippocampus has also been shown to play a role in the modulation of rats' fear reactions (e.g., Trimble, 1988; Treit and Menard, 1997; Cheeta et al., 2000; File et al., 2000), and the hippocampal cholinergic system seems to be particularly important. In general, increases in rats' fear reactions have been observed in a variety of tests following intra-hippocampal infusions of both muscarinic and nicotinic antagonists (Hess and Blozovski, 1987; File et al., 1998a; Smythe et al.,1998; but see Carlton, 1969). Hippocampal cholinergic blockade also enhances the hypothalamic-pituitary-adrenal response to stress (Bhatnagar et al., 1997). Conversely, we showed that facilitation of cholinergic activity using intra-hippocampal infusions of the acetylcholinesterase inhibitor physostigmine reduced fear reactions in plus-maze and shock-probe tests (Degroot et al., 2001; Degroot and Treit, In Press).

Functionally, according to Gray's theory (Gray, 1982; 1991; Gray and McNaughton, 2000), the septum and hippocampus act in concert to control anxiety, as evidenced in part by the remarkable similarity between the effects of septal or hippocampal lesions in traditional aversive learning paradigms and the effects of anxiolytic drugs in the same paradigms (for reviews, see Gray, 1982; Gray and McNaughton, 1983). Structurally, an interaction between the septum and hippocampus in anxiety regulation also seems plausible given the extensive interconnections between the two areas (e.g., Swanson and Cowan, 1977; Nauta and Domesick, 1982; Risold and Swanson, 1997). The hippocampus sends a GABAergic projection to the medial septum and a glutamatergic projection to the lateral septum. The GABAergic projection originates from non-pyramidal cells in the stratum oriens of the CA1-CA3 region and innervates cholinergic and non-cholinergic neurons (Amaral and Witter, 1995). The glutamatergic pathway projects from pyramidal cells and terminates on GABAergic neurons of the lateral septum (Walass and Fonnum, 1980). The latter pathway may be particularly significant since the anxiolytic effects of infusions of midazolam into the hippocampus can be antagonized by co-infusions of glutamate into the lateral septum (Menard and Treit, 2001). Taken together, these data suggest that the hippocampal cholinergic system regulates anxiety through an interaction with the septal GABAergic system.

The purpose of the present study was to provide evidence for a septal-hippocampal interaction in anxiety modulation by stimulating the septal

GABAergic and hippocampal cholinergic systems either independently or simultaneously. To show an interaction, we co-infused sub-effective doses of muscimol and physostigmine in the septum and hippocampus respectively. If the septum and hippocampus act in concert in the control of anxiety, then the sub-effective doses should act synergistically and result in anxiety reduction. However, if the septum and hippocampus do not interact in this way, then simultaneous sub-effective doses should not produce anxiety reduction.

Anxiety was assessed in the shock-probe burying test (for a current review of methods and findings see Treit et al., in press). In this test, rats shocked from a stationary, electrified probe push bedding material from the floor of the experimental chamber toward or over the probe, with rapid alternating movements of the forepaws (i.e., "burying behavior"), while avoiding further contacts with the probe (Pinel and Treit, 1978; Treit et al., 1981; Treit et al., 1994). A reduction in the duration of probe burying, in the absence of a decrease in general activity, is used as the primary index of anxiety reduction (Treit et al., 1981). Standard anxiolytic drugs such as diazepam decrease shock-probe burying (Treit et al., 1981; Tsuda et al., 1988; De Boer et al., 1990; Treit et al., 1993b; Treit et al., 1994), whereas anxiogenic drugs such as yohimbine increase shock-probe burying (Tsuda et al., 1988; Treit, 1990). The suppression of burying by benzodiazepine anxiolytics is not secondary to behavioral sedation (Treit and Fundytus, 1988; Rohmer et al., 1990), associative learning deficits (Blampied and Kirk, 1983), or analgesia (Treit, 1985), and can be reversed by benzodiazepine antagonists such as flumazenil (Treit, 1987). Moreover, probe-shocked rats show increased plasma corticosteroid and adrenaline levels, which can also be blocked by anxiolytic drugs (de Boer et al., 1990). Finally, the shock-probe test does not involve response learning or an explicit memory requirement, factors that can complicate the interpretation of drug effects in other animal models of anxiety (e.g., the conflict test; Treit, 1985). This is particularly important for the current study since both septal GABAergic and hippocampal cholinergic systems have been repeatedly implicated in learning and memory (e.g., Brioni et al., 1990; Durkin, 1992; Degroot and Parent, 2000, 2001).

Materials and Methods

Experiment 1

In experiment 1 we infused different doses of muscimol into the medial septum and examined their effects in the shock-probe burying test. The purpose of this experiment was to characterize the dose-effect relationship of muscimol in this test and, more specifically, to determine effective and sub-effective doses of muscimol.

Subjects

Subjects were male Sprague-Dawley rats (Ellerslie, Canada), weighing 250-300g upon arrival. Following surgery, rats were individually housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle (lights on at 07.00h), with food and water available *ad libitum*. Behavioral testing occurred between 09.00 and 19.00 h. Experiments were carried out in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Surgery and Histology

The rats were given an oral administration of the analgesic acetaminophen (Tylenol 120 mg/1.5 ml; McNeil, Canada) followed one hour later by atropine sulfate (0.1 mg/0.2 ml, i.p.; Ormond Veterinary Supply LTD., Canada) to reduce respiratory complications due to the anaesthetic. The rats were then anaesthetized with pentobarbital (Nembutal 50 mg/kg, i.p.; Abbott Laboratories, Canada), hydrated with saline (3 cc, s.c.), and given the antibiotic penicillin (Crystiben, Rhone Merieux Canada Inc., 15,000 I.U./0.05 cc, i.m.). Stereotaxic procedures were used to implant 60 rats with one 22 gauge stainless-steel guide cannula (Plastics One, Inc. Roanoke, VA) aimed 1 mm above the medial septum (0.5 mm anterior to bregma [AP], 4.9 mm ventral to dura [DV] (Paxinos and Watson, 1986). The incisor bar was adjusted (-3.2 mm) such that bregma and lambda were at the same height. The cannula was attached to the skull with 4 jeweler's screws and cranioplastic cement (Lang Dental MFG. Co., Inc, IL). A dummy cannula was inserted into the guide cannula to keep the cannula tract clear. Immediately after surgery the rats were placed into a warm environment until they regained consciousness. Two days after surgery, each cannula was checked for obstructions, and betadine (Purdue Pharma, Canada) was applied to the surgical wound. Following behavioral testing, rats were sacrificed with an overdose of chloral hydrate (Fisher, Canada) and perfused intracardially with 0.9% saline followed by a 10 % formalin-saline solution (Fisher, Canada). The brains were removed and placed in a 10% formalin-saline solution. After at least 48 hours had elapsed, the brains were frozen with dry ice, sectioned (60 μm), mounted onto glass slides and stained with thionin (Sigma, Canada). The location of the cannula for each rat was examined microscopically by an observer who was "blind" to the behavioral results. The location of the cannula tip was then transcribed onto the appropriate atlas plates (Paxinos and Watson, 1986). The behavioral data for animals with misplaced cannulae or necrosis of brain tissue at the site of implantation were discarded (n = 14). In addition, two rats failed to touch the probe and therefore could not be tested.

Infusion Procedures

Rats were randomly assigned to one of the following conditions: a control condition, infused with vehicle (phosphate-buffered saline; PBS, pH 7.4; 0.4 µl/1 min) or one of three drug conditions, infused with 2.5, 5, or 10 ng muscimol (0.4 µl/1 min; Sigma). Muscimol was dissolved in PBS and the pH adjusted to 7.4. The doses of muscimol were based on previous work in our laboratory (Degroot et al., 2001). The tame, hand-held rats were given an infusion of muscimol or PBS into the medial septum through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula. The internal cannula was connected to a 10 µl constant rate Hamilton microsyringe with polyethylene tubing and the infusions were delivered using an automated infusion pump (Harvard Apparatus 22). The internal cannula was left in place for one minute following the infusions in order to allow for diffusion.

Behavioral Testing

The behavioral testing procedures were the same as those used in previous experiments (e.g., Treit et al., 1993a,b; Pesold and Treit, 1996; Menard and Treit, 2000). All behavior was recorded on videotape for ensuing analysis. All testing occurred at least seven days post-surgery.

The shock-probe burying apparatus consisted of a 40 x 30 x 40 cm plexiglass chamber, evenly covered with approximately 5 cm of bedding material (odor-absorbent kitty litter). The shock-probe was inserted through a small hole on one wall of the chamber, 2 cm above the bedding material. The plexiglass shock-probe (6.5 cm long and 0.5 cm in diameter) was helically wrapped with two copper wires through which an electric current was administered. Current from the shock source (H13-01 2-pole shocker; Coulbourn Instruments) was set at 2 mA. Rats were habituated in pairs to the test chamber without the shock-probe, for 30 minutes on each of four consecutive days prior to the test day. On the test day, five minutes following their respective drug treatments, rats were individually placed in one corner of the testing chamber, facing away from the shock-probe. The probe was not electrified until the rat touched it with its snout or forepaws, at which point the rat received a 2 mA shock. Shock was terminated by the reflexive withdrawal of the rat from the probe. The 15-minute testing period began once the rat received its first shock and the probe remained electrified continuously for the remainder of the testing period. Following the first shock, the duration of time each rat spent spraying bedding material toward or over the probe with its snout or forepaws (i.e. burying behavior) was measured, as was the total number of contact-induced shocks each rat received from the probe. An index of the rat's reactivity to each shock was scored according to the following four-point scale (Pesold and Treit, 1992): (1) flinch involving only head or forepaw, (2) whole body flinch, with or without slow ambulation away from the probe, (3) whole body flinch, and/or jumping, followed by immediate ambulation away from the probe, and (4) whole body flinch and jump (all four paws in the air), followed by immediate and rapid ambulation (i.e. running) to the opposite end of the chamber. A mean shock reactivity score was calculated for each rat by summing its shock reactivity scores and dividing by the total number of shocks it received. The total time that the rat spent immobile (e.g. resting on the floor) during the 15-minute testing period was used as an index of general activity. All behavioral measures were made by a "blind" observer who was watching the rat via a video monitor in a room adjacent to the testing room.

Statistical Analysis

Results are expressed as means and standard errors of the mean (S.E.M.). The shock-probe data were analyzed using analysis of variance (ANOVA) and post-hoc pair-wise comparisons were made using Fisher adjusted least-significant difference (LSD) tests. An alpha level of 0.05 was used as the criterion for statistical significance. In order to correct for non-normality and heterogeneity of variance, the burying and immobility scores were transformed to their natural logarithms prior to ANOVA (for non-transformed scores, see Tables 1-3).

Experiment 2

The purpose of experiment 2 was similar to that of experiment 1: i.e., to characterize the behavioral effects of different doses of intra-hippocampal physostigmine (Sigma), primarily to determine effective and sub-effective doses.

The general procedures and statistical analyses used in this experiment were the same as those used in experiment 1. The same stereotaxic procedures were used to implant 78 rats with one 22 gauge guide cannula aimed 1.2 mm above the dorsal hippocampus (-4.2 mm AP, 2.0 mm DV, 4.1 mm lateral to the midline). Half of the rats (n = 39) had the hippocampal cannulae implanted in the left hemisphere. The behavioral data for animals with misplaced cannulae or necrosis of brain tissue at the site of implantation were discarded (n = 5). In addition, two rats failed to touch the probe, and three rats could not be tested due to difficulties with the infusion procedure. Rats were randomly assigned to one of the following conditions: a control condition, infused with vehicle (phosphate buffered saline; PBS, pH 7.4; 1 µl/1 min) or one of three drug conditions, infused with 5, 10, or 20 *u*g of physostigmine (1 µl/1 min; Sigma). Infusions into the dorsal hippocampus were delivered through a 26 gauge stainless steel internal cannula lowered 1.2 mm below the tip of the guide cannula. The infusions were administered using an infusion pump (Harvard Apparatus 22). The doses of physostigmine were based on previous work (Degroot and Parent 2000; 2001; Degroot et al., 2001, Degroot and Treit, In Press).

Experiment 3

Experiment 3 examined the effects of co-infusing sub-effective doses of physostigmine and muscimol in the hippocampus and septum, respectively. Based on our hypothesis (see introduction), it seemed possible that a synergistic interaction could arise from the simultaneous sub-threshold stimulation of the two systems: i.e. combined sub-threshold infusions should reduce burying behavior. The methods and procedures used in this experiment were basically the same as those used in the first two experiments. Stereotaxic procedures were used to implant 80 rats with one 22 gauge guide cannula aimed 1 mm above the medial septum and one 22 gauge cannula aimed 1.2 mm above the dorsal hippocampus (-4.2 mm AP, 2.0 mm DV, 4.1 mm lateral to the midline). Half of the rats (n = 40) had the hippocampal cannulae implanted in the left hemisphere. The behavioral data for animals with misplaced cannulae or necrosis of brain tissue at the site of implantation were discarded (n = 22). In addition, one rat failed to touch the probe and six rats could not be tested due to problems with the infusion procedure. The rats were given an infusion of muscimol (2.5 ng/0.4 µl/ for 1 min) or vehicle (PBS; 0.4 µl/ for 1 min) into the medial septum through a 26 gauge stainless steel internal cannula lowered 1.0 mm below the tip of the guide cannula, or an infusion of physostigmine (5 µg/1 µl/ for 1 min) or its vehicle (PBS; 1µl/ for 1 min) into the dorsal hippocampus through a 26 gauge stainless steel internal cannula lowered 1.2 mm below the tip of the guide cannula. The infusions were delivered using an infusion pump (Harvard Apparatus 22). The 2.5 ng dose of muscimol was selected based on the findings from experiment 1, while the 5 µg dose for physostigmine was based on the findings from experiment 2. Rats were randomly assigned to one of four drug conditions: (1) vehicle in both the hippocampus and the medial septum, (2) physostigmine in the hippocampus and vehicle in the medial septum, (3) vehicle in the hippocampus and muscimol in the medial septum, or (4) physostigmine in the hippocampus and muscimol in the medial septum.

Results

Experiment 1

Figure 5-1 shows the location of the infusion sites in the medial septum. Figure 5-2 indicates that infusions of muscimol into the medial septum produced anxiolytic-like effects in the shock-probe burying test [$F(3,40) = 3.18$, $p < 0.04$]. Specifically, rats infused with 10 ng muscimol in the medial septum displayed significantly lower burying levels than their vehicle infused controls ($p < 0.01$). This anxiolytic effect occurred in the absence of significant effects on immobility [$F(3,40) = 2.15$, $p > 0.10$; see table 5-1], shock reactivity [$F(3,40) = 0.23$, $p > 0.86$; see table 5-1] or shock-probe contacts [F(3,40) = 2.19, p > 0.09; see table 5-1]. However, intra-septal infusions of 2.5 ng or 5 ng of muscimol did not significantly reduce burying behavior when directly compared to vehicle-infused controls (p > 0.36 and 0.10 respectively).

Experiment 2

Figure 5-3 shows the location of the infusion sites in the dorsal hippocampus. Figure 5-4 indicates that infusions of physostigmine produced anxiolytic-like effects in the shock probe burying test [F(3,64) = 8.56, p < 0.001]. Rats infused with 20 µg of physostigmine in the dorsal hippocampus displayed significantly lower burying levels than their vehicle-infused controls (p < 0.001). This anxiolytic-like effect occurred in the absence of any significant changes in the number of shocks received [F(3,64) = 0.46, p > 0.70; see table 5-2] or shock reactivity [F(3,64) = 2.39, p > 0.07; see table 5-2]. Although there was a significant difference in immobility between groups [F(3,64) = 3.77, p < 0.02; see table 5-2], analysis of covariance showed that immobility (the covariate) did not account for the significant effect of physostigmine on burying behavior [F(1,63 =4.35, p < 0.01]. Intra-hippocampal infusions of 5 µg or 10 µg did not significantly affect burying activity when compared to control (p > 0.30 and 0.17 respectively)

Experiment 3

Figure 5-5 shows that infusion sites in the medial septum and the dorsal hippocampus were similar to those shown in figures 5-1 and 5-3. The behavioral results indicated that sub-threshold doses of muscimol and physostigmine infused into the hippocampus and the septum altered burying behavior in a synergistic fashion

[$F(3,47) = 4.39$, $p < 0.01$]; see Fig. 5-6). Specifically, combined infusions of physostigmine (5 μg) in the hippocampus and muscimol (2.5 ng) in the septum significantly reduced burying behavior compared to control ($p < 0.01$), whereas a single infusion of the same dose of physostigmine in the hippocampus, or a single infusion of the same dose of muscimol in the septum did not affect burying behavior ($p > 0.87$ and 0.99 respectively). The anxiolytic-like effect produced by the combined infusions was not confounded by differences in general activity [$F(3,47) = 1.04$, $p > 0.38$], number of shocks received [$F(3,47) = 1.15$, $p > 0.44$], or shock reactivity [$F(3,47) = 1.68$, $p > 0.17$].

A *post-hoc* analysis of the data obtained from rats with misplaced cannulae (data not shown) indicated that the drug effect was specific to the two target areas (dorsal hippocampus and medial septum). Animals that received drug infusions but had their cannulae misplaced ($n = 5$) displayed normal burying levels compared to vehicle infused controls [$F(1,16) = 1.00$, $p > 0.32$], and had significantly higher burying levels compared to rats that had infusions correctly directed towards the target areas [$F(1,13) = 26.62$, $p < 0.001$].

General Discussion

The results of experiment 1 show that higher doses of muscimol infused into the medial septum tend to suppress burying behavior in the shock probe apparatus, whereas lower doses do not. This finding is consistent with a previous study by Drugan and colleagues, 1986, who demonstrated that infusions of increasing doses of muscimol into the lateral septum produced a linear increase in punished responding in a conflict test. The results of experiment 1 also replicate and extend a previous study in our laboratory, which indicated that intra-septal infusions of a single dose of muscimol have anxiolytic-like effects (Degroot et al., 2001). The findings of experiment 2 demonstrate that infusions of physostigmine into the dorsal hippocampus also modulate anxiety levels in the shock-probe burying test. The highest dose of physostigmine significantly suppressed burying behavior, whereas the lower doses did not. The anxiolytic-like effect we found in experiment 2 is consistent with previous findings: i.e., intra-hippocampal infusions of cholinergic *agonists* decrease anxiety (File et al., 1998b; Degroot et al., 2000; File et al., 2000; Degroot and Treit, in press), while intra-hippocampal infusions of cholinergic *antagonists* increase anxiety (Hess and Blozovski, 1987; File et al., 1998a; Smythe et al., 1998). More importantly, the results from experiment 3 indicate that the hippocampal cholinergic and septal GABAergic systems can interact synergistically to modulate anxiety levels. Specifically, simultaneous infusions of sub-effective doses of physostigmine in the hippocampus and muscimol in the medial septum summate to produce a significant reduction of burying behavior.

It seems unlikely that these anxiolytic-like effects can be explained in terms of non-specific effects on shock-reactivity, or shock-probe avoidance since neither of these measures was significantly affected in any of our experiments. In one instance (Experiment 2), rats did differ in general activity, but when this effect was statistically controlled (ANCOVA), the suppression of burying behavior by physostigmine was still significant. It is also unlikely, given our small infusion volumes, that the effects we found were due to drug diffusion away from intended target sites. Thus, our study may be the first to provide clear evidence that hippocampal cholinergic and septal GABAergic systems can interact to reduce anxiety.

The neural circuitry that underlies the synergistic interaction we found between the hippocampal cholinergic and septal GABAergic systems remains to be determined. One hypothesis is that the medial septum, rather then the hippocampus, is the nodal point at which septo-hippocampal pathways converge to modulate anxiety. According to this hypothesis, both hippocampal infusions of physostigmine and septal infusions of muscimol should ultimately have the same effect: i.e., a suppression of medial septal neurons. The hippocampus could inhibit the medial septum through either a direct or an indirect pathway. The direct pathway is the GABAergic projection from the hippocampus to the medial septum (Amaral and Witter, 1995). The indirect pathway is the excitatory glutamatergic projection from the hippocampus to the lateral septum, which in turn stimulates an inhibitory GABAergic projection to the medial septum (Alonso and Köhler, 1982; Waine, 1985; Freund and Antal, 1988; Kiss et al., 1990; Nauman et al., 1992; Toth et al., 1993). Thus, stimulating hippocampal cholinergic receptors could excite the direct hippocampal GABAergic projection, or the indirect glutamatergic projection. In either case, GABAergic inhibition of the medial septum would be achieved.

While the scenario proposed above might be consistent with the anxiolytic effect of muscimol we found in the medial septum (Experiment 1), and the anxiolytic effect of physostigmine we found in the dorsal hippocampus (Experiment 2), we have no direct evidence that this particular circuitry actually mediated the effects we found in these experiments. Furthermore, it does not address the question of how *sub-*

effective doses of muscimol and physostigmine could summate to produce an anxiolytic effect (Experiment 3), although it is possible the same circuitry might have been recruited. Thus, at this point, the functional circuitry between hippocampal cholinergic and septal GABAergic systems outlined above is best viewed as a working hypothesis for future studies.

Although the overall pattern of our results is consistent with Gray's general theory of septo-hippocampal function, it does not necessarily accommodate all of the details of this theory or its subsequent elaborations (Gray and McNaughton, 2000). For example, Gray's 1982 model suggests that the cholinergic contribution proceeds from the septum to the hippocampus, while our data suggest that the cholinergic contribution proceeds from the hippocampus to the septum. On the other hand, Gray's model does not explicitly rule out this alternative route, or that both routes could be operative in the control of anxiety. Another aspect of Gray's model is that hippocampal theta may be involved in the septo-hippocampal control of anxiety. However, since our septal GABAergic and hippocampal cholinergic manipulations likely have opposite effects on theta rhythm (Allen and Crawford, 1984; Dutar et al., 1995), it would seem that the anxiolytic effects we found in the present experiments are independent of hippocampal theta. In spite of these complexities, however, our view that the medial septum is a nodal point in the control of anxiety is broadly in line with Gray's model.

More importantly, on an empirical level, our results strongly suggest that the hippocampal cholinergic and septal GABAergic systems interact in a synergistic manner to modulate anxiety. Furthermore, our findings provide detailed and specific empirical support for Gray's general theory that the septum and the hippocampus act in concert in the regulation of anxiety. Although the exact pathways through which the hippocampus and septum interact to modulate anxiety remain to be determined, the finding that intra-septal glutamate can reverse the anxiolytic effects of intra-hippocampal midazolam (Menard and Treit, 2001) offers an interesting strategy in this regard. These findings suggest that inhibition of the efferent pathways of the hippocampus, along with simultaneous stimulation of target structures such as the septum, may ultimately delineate the specific circuitry through which the hippocampus and septum interact to control anxiety.

Table 5-1. Mean (+/- S.E.M.) activity, reactivty, and burying scores in the shock-probe burying test.

	Vehicle	Muscimol	Muscimol	Muscimol
	(n = 12)	2.5 ng (n = 11)	5.0 ng (n = 9)	10.0 ng (n =12)
Burying time (s)	91.67	33.00	38.22	10.58
	(36.76)	(11.10)	(22.72)	(4.58)
Immobility (s)	80.17	34.18	161.56	18.50
	(38.65)	(21.24)	(78.31)	(6.96)
Shock reactivity	2.13	2.15	2.23	2.28
	(0.15)	(0.20)	(0.10)	(0.11)
Shock number	2.58	1.82	2.33	1.83
	(0.26)	(0.18)	(0.37)	(0.24)

Table 5-2. Mean (+/- S.E.M.) activity, reactivity, and burying scores in the shock-probe burying test.

	Vehicle (n = 19)	Physostigmine 5 μg (n=15)	Physostigmine 10 μg (n = 18)	Physostigmine 20 μg (n = 17)
Burying time (s)	45.32	62.00	31.50	2.25
	(13.66)	(13.88)	(13.15)	(1.05)
Immobility (s)	70.53	49.67	20.61	56.69
	(23.58)	(16.58)	(6.58)	(14.42)
Shock Reactivity	1.82	2.05	1.74	1.52
	(0.15)	(0.11)	(0.13)	(0.14)
Shock number	2.05	1.87	2.22	2.00
	(0.14)	(0.19)	(0.26)	(0.24)

Table5-3. Mean (+/- S.E.M.) activity, reactivity, and burying scores in the shock-probe burying test.

	PBS/PBS	PHYS/PBS	PBS/MUSC	PHYS/MUSC
	(n = 13)	(n = 17)	(n = 11)	(n = 10)
Burying time (s)	46.23	55.41	55.45	3.00
	(12.84)	(16.56)	(16.63)	(1.93)
Immobility (s)	44.92	23.24	96.00	138.60
	(17.57)	(9.67)	(42.09)	(71.69)
Shock reactivity	1.71	2.14	2.16	2.22
	(0.15)	(0.15)	(0.15)	(0.29)
Shock number	1.77	1.82	1.45	2.10
	(0.26)	(0.20)	(0.21)	(0.23)

Figure 5-1. Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septal infusion sites in Experiment 1. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from Paxinos and Watson (1986). The histological maps exclude rats that were eliminated from the experiment.

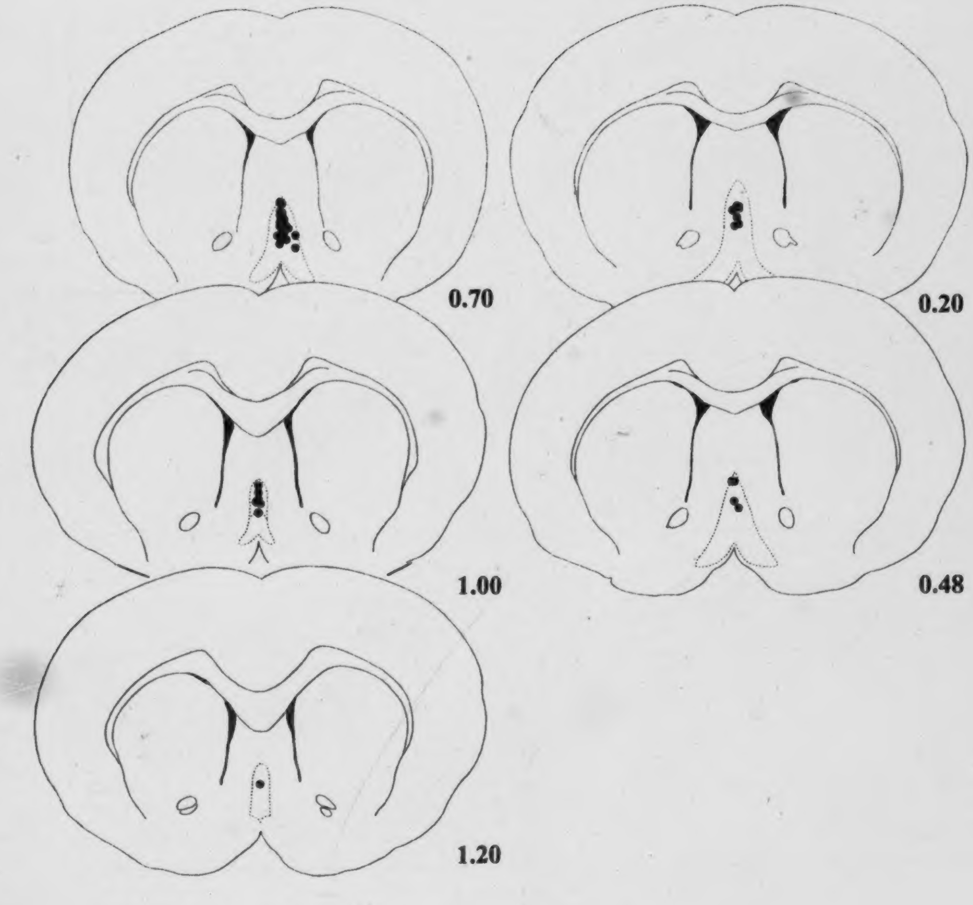

0.70
0.20
1.00
0.48
1.20

Figure 5-2. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of muscimol (2.5, 5, or 10 ng/0.4 μl) or vehicle (0.4 μl) into the medial septum. *p < .01 compared with the vehicle control group.

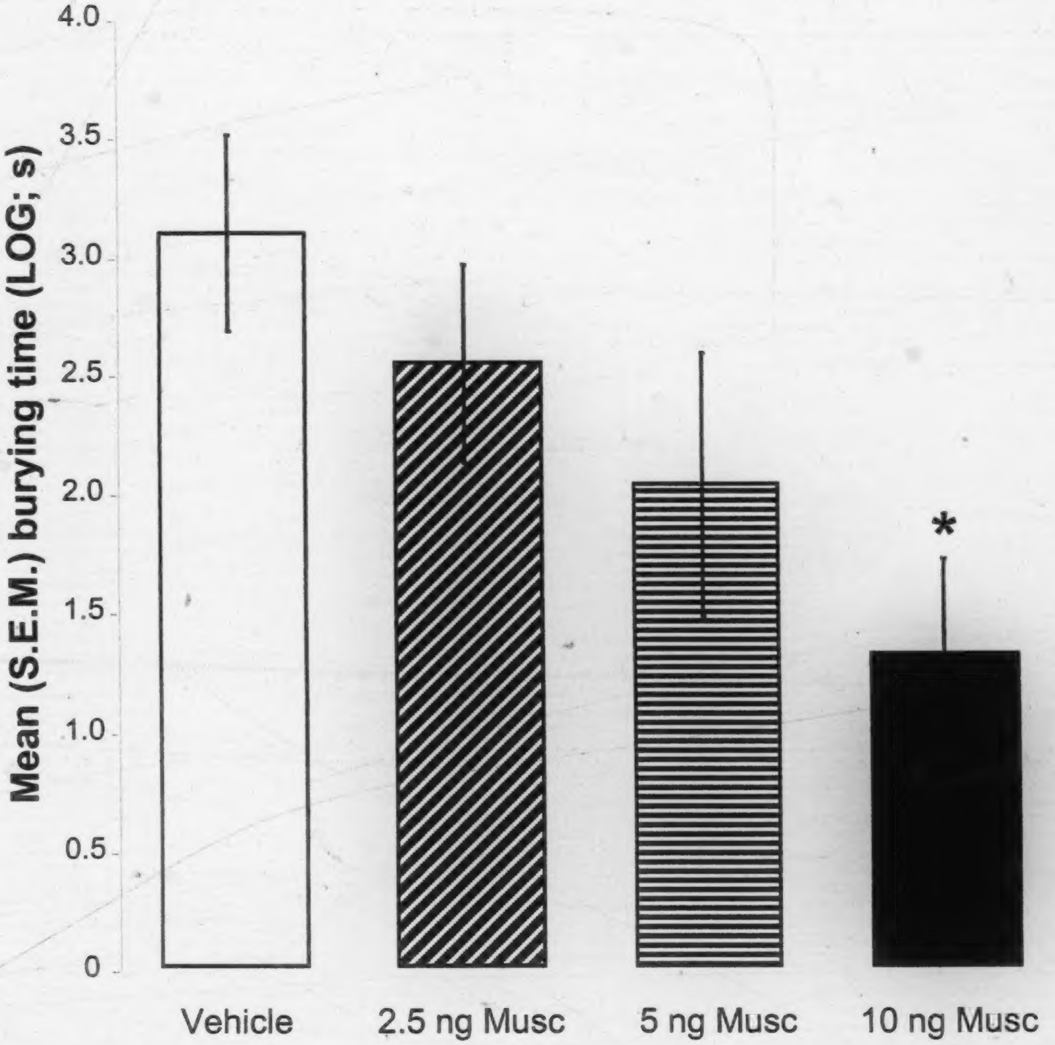

Mean (S.E.M.) burying time (LOG; s)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Vehicle
2.5 ng Musc
5 ng Musc
10 ng Musc

Figure 5-3. Schematic illustration of coronal sections of the rat brain showing the approximate location of dorsal hippocampal infusion sites in Experiment 2. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from Paxinos and Watson (1986). The histological maps exclude rats that were eliminated from the experiment.

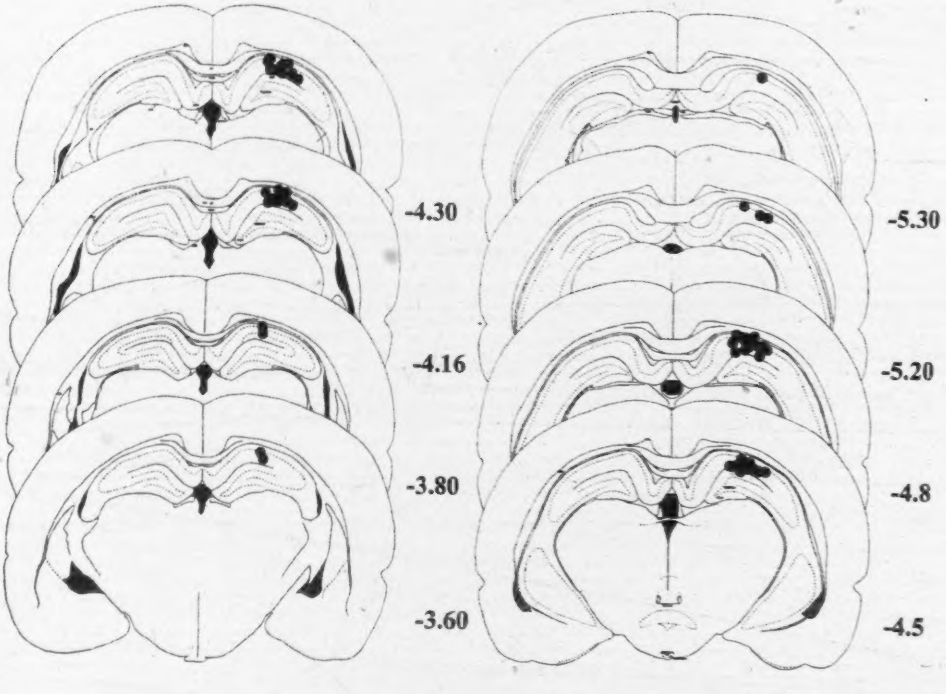

-4.30
-4.16
-3.80
-3.60
-5.30
-5.20
-4.8
-4.5

Figure 5-4. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of physostigmine (5, 10, or 20 μg/1 μl) or vehicle (1 μl) into the dorsal hippocampus. *$p < .001$ compared with the vehicle control group.

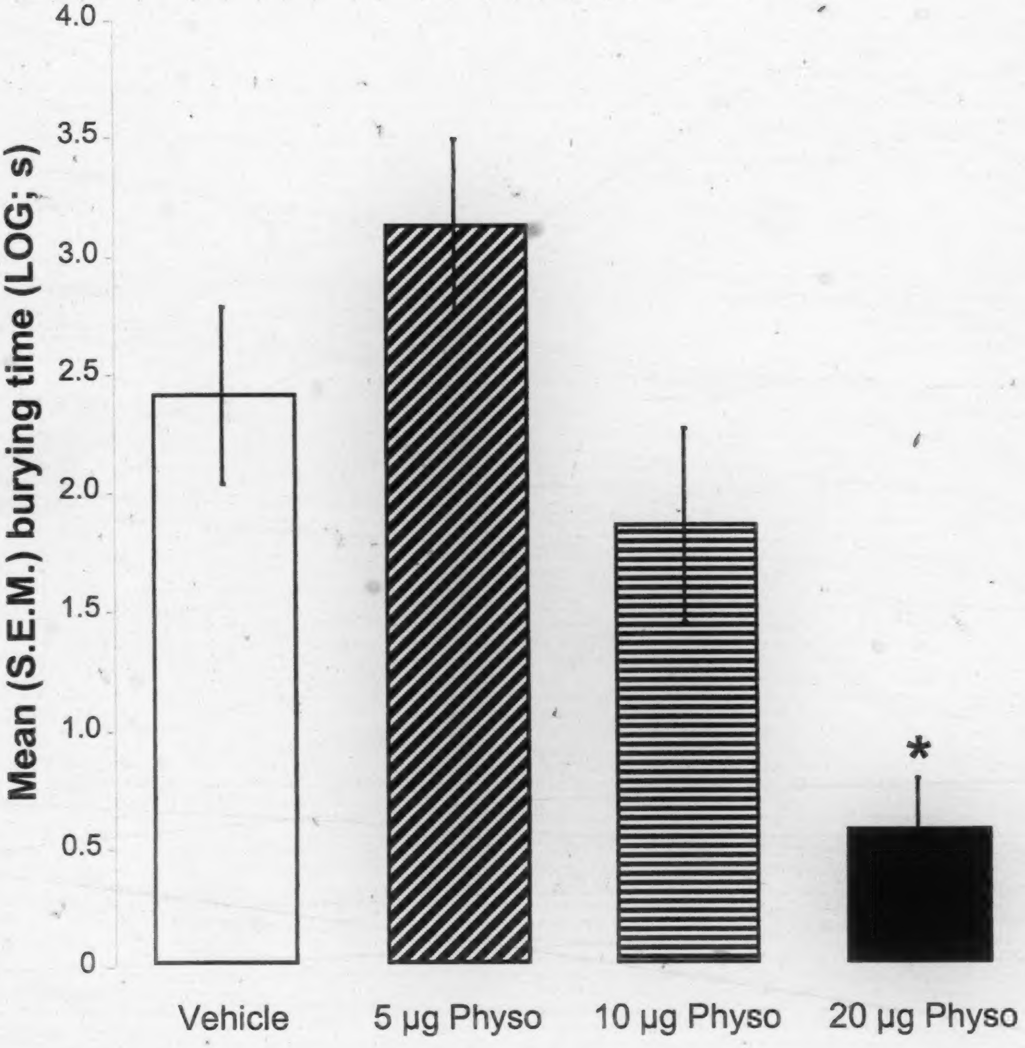

Mean (S.E.M.) burying time (LOG; s)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Vehicle
5 µg Physo
10 µg Physo
20 µg Physo

Figure 5-5. Schematic illustration of coronal sections of the rat brain showing the approximate location of medial septum (A) and dorsal hippocampal (B) infusion sites in Experiment 3. The numbers indicate A-P coordinates relative to Bregma. Atlas plates adapted from Paxinos and Watson (1986). The histological maps exclude rats that were eliminated from the experiment.

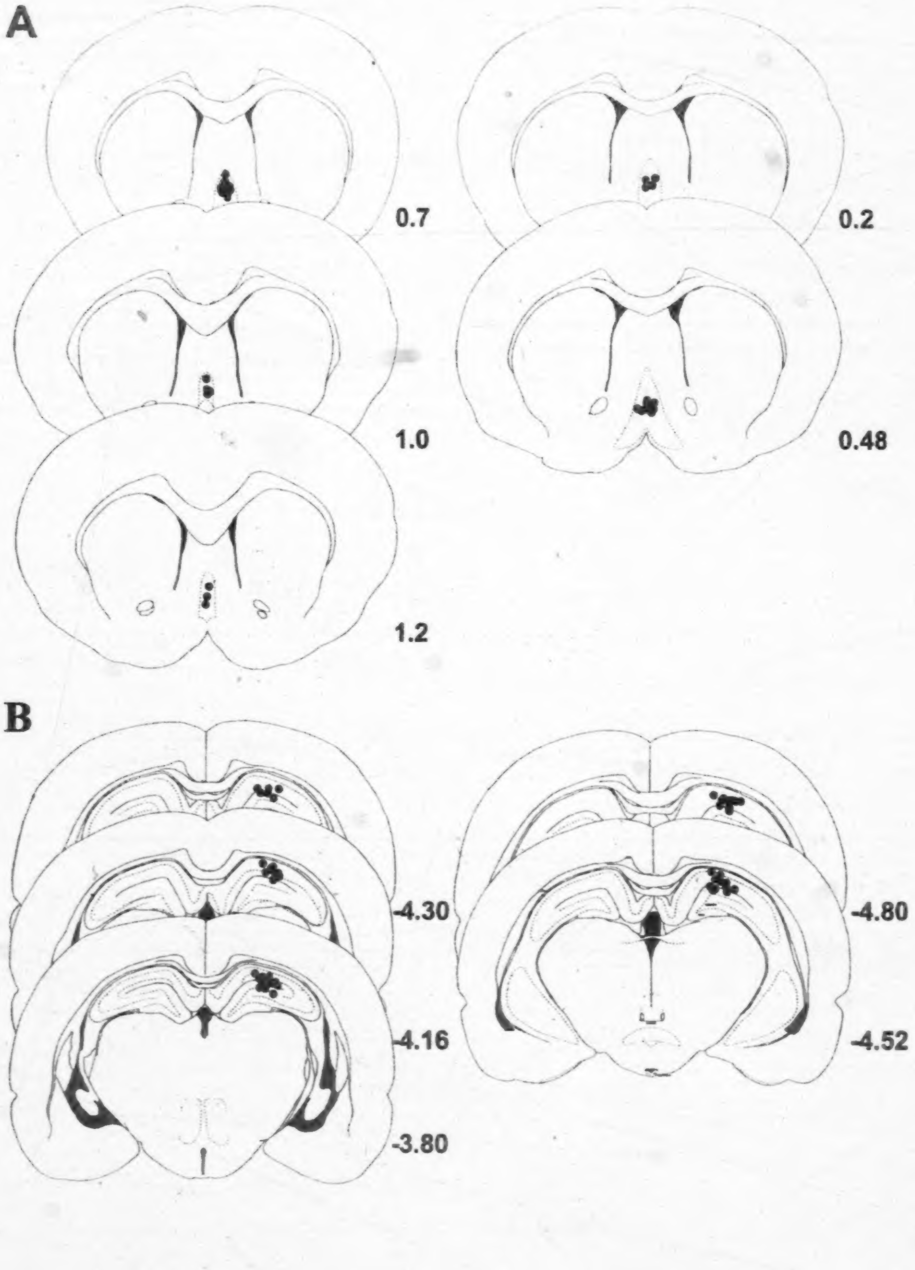
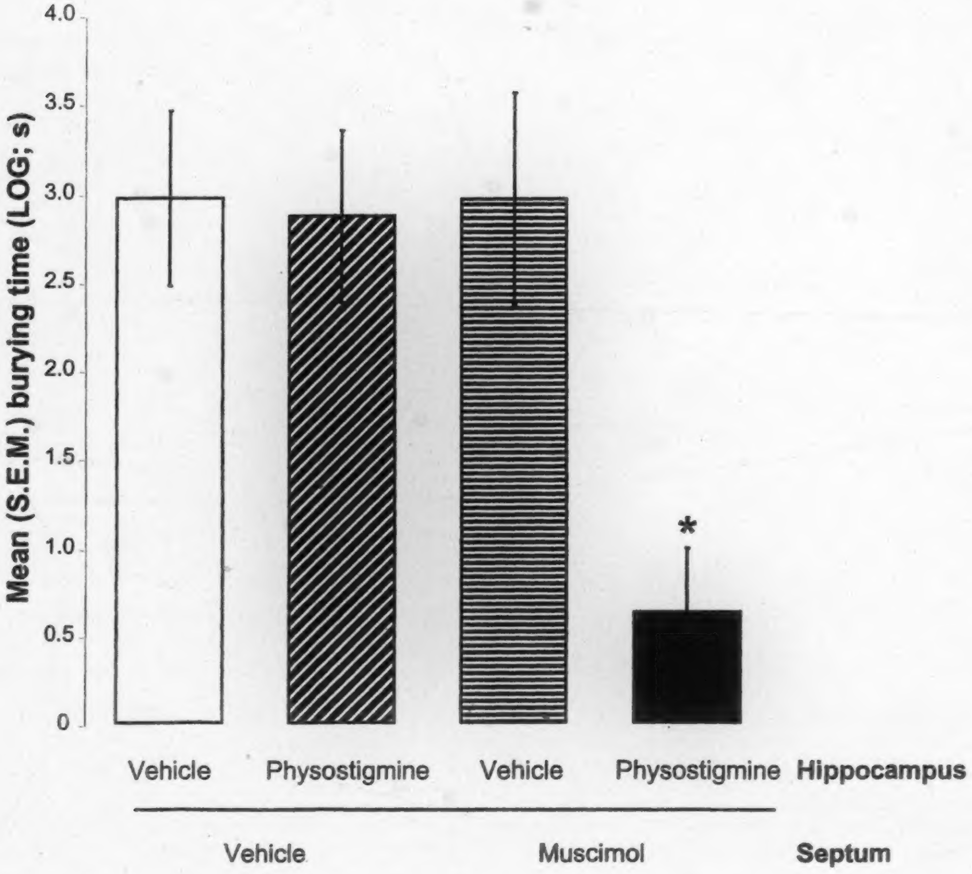

A
0.7
0.2
1.0
0.48
1.2
B
-4.30
-4.80
-4.16
-4.52
-3.80

Figure 5-6. Mean (+/- S.E.M.) bury time (LOG; s) in the shock probe apparatus after infusions of muscimol (2.5 ng/0.4 ul) into the medial septum, physostigmine (5 μg/1 μl) into the dorsal hippocampus, or combined infusions. *p < .01 compared with the vehicle control group.


Mean (S.E.M.) burying time (LOG; s)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Vehicle
Physostigmine
Vehicle
Physostigmine
Hippocampus
Vehicle
Muscimol
Septum

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Chapter 6

Discussion

General Discussion

One issue raised by the current studies is the extent to which the septum and hippocampus exert distinct or common control over rats' anxiety reactions. For example, our first set of studies (Chapter 2) indicated that TTX lesions of the ventral hippocampus or the medial septum increased open-arm exploration in the plus-maze and decreased burying behavior in the shock-probe test, but neither manipulation affected contacts made with the probe. In contrast, dorsal hippocampal and fimbria fornix lesions increased shock-probe contacts but had no effect on burying behavior. Furthermore, like ventral hippocampal and medial septal lesions, but unlike dorsal hippocampal lesions, fimbria fornix lesions increased open arm exploration in the plus-maze. In our second set of experiments (Chapter 3), a neurochemical dissociation was found between the dorsal and ventral hippocampus in anxiety modulation. Increasing cholinergic function in either the dorsal or the ventral hippocampus increased open arm exploration in the plus-maze and decreased burying behavior in the shock-probe test. However, increasing cholinergic activity in the ventral, but not the dorsal, hippocampus also increased contacts made with the probe.

While these findings suggest that the control of anxiety is functionally segregated within the septo-hippocampal system, it is important to note that neither anatomical nor neurochemical dissociations were found within the septal region. Data from our third set of experiments (Chapter 4) indicated that stimulating GABAergic receptors in either the medial or the lateral septum had the same effect on anxiety. Either manipulation mimicked TTX lesions of the septum, i.e., open arm exploration was increased, whereas burying behavior was decreased (see Table 6-

1). Neither manipulation affected shock-probe avoidance.

Regardless of whether the septum and the hippocampus exert distinct or common control on rats' anxiety reactions, a second issue is whether these structures can interact cooperatively in the control of specific anxiety reactions. According to Gray's (1982) original theory of septo-hippocampal function, the septum and the hippocampus act in concert to control anxiety. This could imply that either 1) the septum and the hippocampus are functionally homogeneous in their control of anxiety, or 2) separate "nodes" within this system interact in the modulation of anxiety. Taken together, the results of the first set of experiments suggest that the septum and the hippocampus do not simply act in a homogeneous fashion in the control of anxiety. Numerous behavioral dissociations were found after selective lesions of different aspects of the septo-hippocampal system. Furthermore, lesions of the fimbria fornix, a major pathway connecting the two structures, reduced anxiety levels in both the plus-maze and shock-probe tests (see Table 1). The latter result suggests that an uninterrupted communication between the septum and the hippocampus is necessary for the expression of certain fear responses. It also suggests that the septum and the hippocampus may interact in a cooperative fashion in the control of specific anxiety reactions. In this regard, in our last set of studies (Chapter 5), we found that septal GABAergic and hippocampal cholinergic systems seem to act synergistically in the control of anxiety. Specifically, combined sub-effective doses of muscimol in the septum and physostigmine in the hippocampus resulted in decreased anxiety in the shock-probe test (see Table 1).

As mentioned, results from the second set of studies indicated that infusions of physostigmine in the ventral, but not the dorsal, hippocampus significantly impaired passive avoidance of the probe. Since amygdala lesions result in an impairment of probe avoidance in the shock-probe apparatus (Treit and Menard, 1997; Treit et al., 1993), this raises the possibility that the ventral hippocampal effect may have been mediated through the amygdala. Evidence consistent with this possibility was found in the first set of studies, where anterograde tracing studies confirmed that the ventral, but not the dorsal, hippocampus has extensive connections with the amygdala. Previous studies have shown that this pathway is likely peptidergic in nature (Amaral and Witter, 1995). It can be speculated that stimulating ventral hippocampal cholinergic receptors could ultimately have an inhibitory effect on the amygdala through this peptidergic pathway.

Thus far the role of the hippocampal cholinergic system in anxiety regulation remains unclear. One theory proposes that the hippocampal cholinergic system may act as a coping mechanism. Hippocampal cholinergic levels may increase during stressful situations to help the organism cope with fear provoking stimuli, thereby decreasing anxiety (Smythe et al., 1998). An alternative possibility is that the hippocampal cholinergic system modulates anxiety through the hypothalamic-pituitary-adrenal axis (Bhatnagar et al., 1997). A third possibility is that stimulating hippocampal cholinergic receptors modulates anxiety through an interaction with other neurotransmitter systems (File et al., 2000; Kenny et al., 2000). Finally, a fourth possibility is that the hippocampal cholinergic system may modulate anxiety through an effect on septal GABAergic levels, a mechanism that was suggested in the final set of experiments. It may ultimately be determined that the hippocampal cholinergic system may modulate anxiety through an interaction of all four of these possibilities.

Limitations

All of the current studies used the intracerebral infusion technique. Although a powerful tool for determining specific contributions made by various neurotransmitters and brain regions to specific behavioral functions, it is not without potential drawbacks. The interpretation of drug effects can be complicated by unintended effects such as changes in osmolarity, pH, and drug diffusion (Menard and Treit, 1999). These complications were limited somewhat by using small volumes (0.4 –1.0 ul) and by maintaining a steady pH of 7.4.

The first set of studies indicated that fimbria fornix lesions impaired both open-arm avoidance in the plus-maze and shock-probe avoidance in the shock-probe burying test. This raises the possibility that the apparent effects on anxiety observed after fimbria fornix lesions were the result of a non-specific impairment in behavioral inhibition. However, this interpretation seems unlikely given that neither the general activity nor the burying behavior of fimbria fornix-lesioned rats was significantly different from controls. Nevertheless, it is possible that fimbria fornix lesions did result in a selective impairment of passive avoidance. In addition, it was anticipated that stimulating GABAergic receptors in the medial and lateral septum would have distinct effects on anxiety, as was found in a previous study (Pesold and Treit, 1996). In the study by Pesold and Treit, anxiolytic effects were found when the indirectly acting GABA$_A$ agonist midazolam was infused into the lateral septum but not after medial septal infusions. Perhaps the reason for this difference is that muscimol is a directly acting $GABA_A$ agonist and therefore more potent than midazolam. In this regard, septal infusions of muscimol may be more like septal lesions. Previous work has shown that lesions of either the medial or the lateral septum decrease anxiety measures in the plus-maze and shock-probe tests (Menard and Treit, 1996).

Although it was hypothesized that the septum and hippocampus might interact in anxiety regulation, the nature of the effect was different than anticipated. In previous studies using animal models of learning and memory, stimulating hippocampal cholinergic systems reversed the memory-impairing effect of septal muscimol infusions (Degroot and Parent, 2000, 2001). Thus it was anticipated that a similar effect would be observed in animal models of anxiety. Instead, the opposite was seen, in that simultaneously stimulating the septal GABAergic and hippocampal cholinergic systems had additive effects on anxiety modulation (Chapter 5). Overall, these results may not be surprising since the present studies showed that stimulating either system reduces anxiety measures. Also, the fact that the data did not mirror the effects obtained in learning and memory experiments seems to rule out a learning and memory involvement in the present studies.

Lastly, a septo-hippocampal interaction was not observed in the third set of experiments (Chapter 4), but was observed in the final set of studies (Chapter 5). The reason for this inconsistency remains unclear, but may suggest that cholinergic and GABAergic receptors were already saturated in the third set of experiments, and that specific doses need to be used to observe the interaction. In fact, the notion that specific doses need to be used to observe an interaction is supported by pilot data. In the pilot studies, a 5 ng dose of muscimol instead of a 2.5 ng dose was infused into the septum in combination with 5 ug physostigmine into the hippocampus. Although this combination of doses also resulted in suppressed burying behavior in the shock-probe test, the infusion of this higher muscimol dose appeared to have an effect on its own. Therefore, a lower dose of muscimol had to be used in order to show an interaction (Chapter 5). However, open arm exploration in the plus-maze test was not affected when the same set of doses was used (2.5 ng of muscimol in combination with 5 ug of physostigmine). Although this could suggest that the summation effect is task-specific, a more likely possibility is that one needs to use a particular combination of doses to observe the effect across different fear reactions. Pilot data obtained in the plus-maze and shock-probe tests with infusions of muscimol in the septum and physostigmine in the hippocampus indicate that doses that are effective in shock-probe may not necessarily be effective in plus-maze. Thus, it appears possible that slightly different doses of muscimol and physostigmine would have to be used to demonstrate an interaction in the plus-maze test of anxiety.

Future Directions and Conclusions

From the experiments in this dissertation as well as previous studies it is evident that more data need to be gathered regarding the role of the septo-hippocampal system in anxiety regulation. One issue that needs to be further explored is the role of the hippocampal cholinergic system in the control of anxiety. As mentioned, there are different theories on how increasing hippocampal cholinergic levels reduce anxiety. One set of experiments could test the possibility that the hippocampal cholinergic system modulates anxiety through the hypothalamic-pituitary-adrenal axis by measuring the release of stress hormones while increasing hippocampal cholinergic levels. A second set of studies could use microdialysis to explore the possibility that increasing hippocampal ACh might modulate anxiety by increasing other neurotransmitters within the hippocampus (e.g., serotonin and/or GABA). Microdialysis could also be used to directly determine the effect of increasing hippocampal cholinergic levels on septal GABAergic levels in the behaving rat. This experiment would directly test the hypothesis proposed in this dissertation that increasing hippocampal cholinergic levels may modulate anxiety through an effect on septal GABAergic systems (Chapter 5). Furthermore, using microdialysis to measure hippocampal cholinergic levels and septal GABAergic levels while the animal is behaving in plus-maze or shock-probe would provide further insight into the behavioral relevance of these neurochemical events.

The work in this dissertation could be further extended with studies that explore the roles of the muscarinic and nicotinic receptor types in the dorsal and ventral hippocampus. Physostigmine could be infused in the dorsal or ventral hippocampus in combination with specific muscarinic and nicotinic antagonists immediately prior to measuring anxiety in the elevated plus-maze or shock-probe tests. Another set of future studies could combine lesion techniques with intracranial drug infusions. More specifically, the fimbria fornix could be lesioned while increasing hippocampal cholinergic levels. If increasing hippocampal cholinergic levels reduces anxiety through an effect on the septum, then increasing hippocampal cholinergic levels while the fimbria fornix is lesioned should have no effect on anxiety. A similar set of studies could examine the possibility that increasing ventral hippocampal cholinergic levels impairs passive avoidance of the probe through an effect on the amygdala. Ventral hippocampal cholinergic levels could be increased while the pathway between the ventral hippocampus and the amygdala is lesioned. If the ventral hippocampal cholinergic system modulates passive avoidance of the probe through an effect on the amygdala, then increasing ventral hippocampal cholinergic levels while the pathway is lesioned should have no effect on probe avoidance.

Thus, the current dissertation provides an important interface between previous and future experiments. The present experiments extended previous studies that examined the role of the septo-hippocampal system in anxiety regulation and set the stage for future experiments that could be done. In addition to providing general support for the theory proposed by Gray (1982) the present findings further refined and extended Gray's work. The studies also reconfirmed the concept of functional segregation in anxiety regulation. Lastly, further evidence for the role of the hippocampal cholinergic system in anxiety regulation was found.

Table 6-1. Summary table.

Chapter-Experiment	Drug	Effective Dose	Site	Plus-Maze effect	Shock-Probe effect
2-1	TTX	5 ng	FFX	Anxiolytic	↑contacts
2-2	TTX	5 ng	MS	Anxiolytic	↓ burying
2-3	TTX	5 ng	VH	Anxiolytic	↓ burying
2-4	TTX	5 ng	DH	Nil	↑contacts
3-1	Physo.	10 ug	DH	Anxiolytic	↓ burying
3-2	Physo.	10 ug	VH	Anxiolytic	↓ burying, ↑contacts
4-1	Musc.	20 ng	MS or LS	Anxiolytic	↓ burying
5-1	Musc.	10 ng	MS	Anxiolytic	↓ burying
5-2	Physo.	20 ug	DH	Anxiolytic	↓ burying
5-3	Musc. and Physo.	Combined sub-effective doses were used	DH (Physo.), MS (musc.)	Not tested	↓ burying

Physo.: Physostigmine
Musc.: Muscimol
FFX: fimbria fornix
MS: medial septum
LS: lateral septum
DH: dorsal hippocampus
VH: ventral hippocampus

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